DECLARATION UNDER 37 CFR 132

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re: patent application of:

Serial No: 10/563,094	Examiner: Haq, Shafiqul
Filed: (as International Application PCT/GB04/02807) 1 July 2004	Art Unit: 1641
For: Saini et al	Docket No: P08828US00/BAS

DECLARATION UNDER 37 CFR'132

Commissioner for Patents
PO Box 1450
Alexandra, VA 22313-1450

SIR:

I, David C. Cullen, hereby declare:

1) I am a Professor of Bioanalytical Technology at Cranfield University, Cranfield MK43 0AL, United Kingdom.

2) My formal training is in biochemistry. I have a BSc Honours degree in biochemistry from the University of East Anglia (England) and a PhD from the University of Cambridge (England), where I also carried out post-doctoral studies. I moved to Cranfield University in 1994 initially as a lecturer in biophysics and biosensors. The production of small-molecule protein conjugates using spacer molecules is an important part of my current work that involves the development of antibodies to small-molecules and which would be indicative of Life on Mars. This forms part of a large international project to

develop an antibody-based instrument to go on a European Space Agency rover mission (ExoMars) to Mars to search for evidence of Life.

3) I have read the specification of the present application and the office action dated 30 March 2009.

4) I note that in paragraphs 5 and 6 of the office action the examiner alleges that it is unclear what compounds are encompassed by the terms "spacer" and "group capable of binding to a carrier protein" in claim 1.

5) From my knowledge of the field, I am aware that procedures for attaching small molecules to proteins so that antibodies can be produced have been very well known for many years. It is common for there to be a moiety termed a "spacer" interposed between the small molecule and the protein. This commonly improves the ability of the conjugate to elicit production of antibodies that are specific to the small molecule (often called the hapten when described in context of antibody production). The nature of the spacer generally has some effect on this ability. However, it is an entirely routine matter, well within the capacity of scientists in the field, to produce conjugates containing a range of spacers and screen them for suitability.

6) The book "Bioconjugate Techniques" published by Elsevier in 1996 (ISBN: 978-0-12-342335-1) is a compilation of known techniques for producing "bioconjugates" including hapten-protein conjugates. I attach as Annex A the inside front cover (giving bibliographical information); as Annex B, Chapter 9 entitled "Preparation of hapten-carrier immunogen conjugates" and as Annex C, Chapter 5, entitled "Heterobifunctional cross-linkers.

7) Chapter 9 is specifically about the preparation of hapten-carrier immunogen conjugates and shows that this was a common procedure by 1996. The use of proteins as carriers is discussed on pages 421-427. There are many references to the use of spacers, e.g. page 441, lines 4 and 6, and page 443, 2 lines from foot of main text. There is also extensive discussion of suitable groups capable of binding to the carrier protein, particularly in Section 6 beginning on page 446, Section 7 beginning on page 453 and Section 8 beginning on page 454.

8) Chapter 5 contains extensive discussion of the choice of a "cross-bridge or spacer", e.g. on page 229, lines 10-15.

9) This supports my belief that scientists in the field are very familiar with procedures for attaching molecules to proteins, and it is an entirely routine matter to select a suitable "group capable of binding to a protein", with due regard to the particular protein being used, and the nature of the molecule to be attached to it.

10) I hereby declare that all the statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 8 of the United States Code and that such willful statements may jeopardize the validity of the application or any patent issued thereon.

Respectfully submitted,

Date: _____ _____

David C. Cullen

ANNEX A

Front Cover Illustration: A DNA double helix chemically modified at the N2 of a guanine residue to possess a γ-aminobutyric acid (GABA) group. The molecular model was kindly provided by Dr. George Pack of the University of Illinois College of Medicine at Rockford.

This book is printed on acid-free paper. ♾

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ANNEX B

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Heterobifunctional Cross-linkers

Heterobifunctional conjugation reagents contain two different reactive groups that can couple to two different functional targets on proteins and other macromolecules (Fig. 155). For example, one part of a cross-linker may contain an amine-reactive group, while another portion may consist of a sulfhydryl-reactive group. The result is the ability to direct the cross-linking reaction to selected parts of target molecules, thus garnering better control over the conjugation process.

Heterobifunctional reagents can be used to cross-link proteins and other molecules in a two- or three-step process that limits the degree of polymerization often obtained using homobifunctional cross-linkers (Chapter 1, Section 1.2, and Chapter 4, Section 2.2). In a typical conjugation scheme, one protein is modified with a heterobifunctional using the cross-linker's most reactive or most labile end. The modified protein is then purified from excess reagent by gel filtration or rapid dialysis. Most heterobifunctionals contain at least one reactive group that displays extended stability in aqueous environments, therefore allowing purification of an activated intermediate before adding the second molecule to be conjugated. For instance, an NHS ester–maleimide heterobifunctional (for example, see this chapter, Section 1.3) can be used to react with the amine groups of one protein through its NHS ester end (the most labile functional group), while preserving the activity of its maleimide functional group. Since the maleimide group has greater stability in aqueous solution than the NHS ester group, a maleimide-activated intermediate may be created. After a quick purification step, the maleimide end of the cross-linker then can be used to conjugate to a sulfhydryl-containing molecule.

Such multistep protocols offer greater control over the resultant size of the conjugate and the molar ratio of components within the cross-linked product. The configuration or structure of the conjugate can be regulated by the degree of initial modification of the first protein and by adjusting the amount of second protein added to the final conjugation reaction. Thus, low- or high-molecular-weight conjugates may be obtained to better fashion the product toward its intended use.

Heterobifunctional cross-linking reagents also may be used to site-direct a conjugation reaction toward particular parts of target molecules. Amines may be coupled on one molecule while sulfhydryls or carbohydrates are targeted on another molecule. Directed coupling often is important in preserving critical epitopes or active sites within macromolecules. For instance, antibodies may be coupled to other proteins

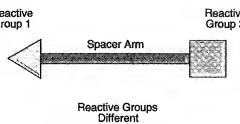


Figure 155 The general design of a heterobifunctional cross-linking agent includes two different reactive groups at either end and an organic cross-bridge of various length and composition. The cross-bridge may be constructed of chemically cleavable components for selective disruption of conjugates.

while directing the cross-linking reaction away from the antigen binding sites, thus maximizing antibody activity in the conjugate.

Heterobifunctional reagents containing one photoreactive end may be used to nonselectively insert into target molecules by UV irradiation. Ligands having specific affinity toward a receptor may be labeled with a photoreactive cross-linker, allowed to interact with its target, and then photolyzed to label permanently the receptor at its binding site. The photoreactive group is stable until exposed to high intensity light at UV wavelengths. Photoaffinity labeling techniques are an important investigative tool for determining binding site characteristics.

The third component of all heterobifunctional reagents is the cross-bridge or spacer that ties the two reactive ends together. Cross-linkers may be selected based not only on their reactivities, but also on the length and type of cross-bridge they possess. Some heterobifunctional families differ solely in the length of their spacer. The nature of cross-bridge also may govern the overall hydrophilicity of the reagent. A number of heterobifunctionals contain cleavable groups within their cross-bridge, lending greater flexibility to the experimental design. A few cross-linkers contain peculiar cross-bridge constituents that actually affect the reactivity of their functional groups. For instance, it is known that a maleimide group that has an aromatic ring immediately next to it is less stable to ring opening and loss of activity than a maleimide that has an aliphatic ring adjacent to it. In addition, conjugates destined for use *in vivo* may have different properties depending on the type of spacer on the associated cross-linker. Some spacers may be immunogenic and cause specific antibody production to occur against them. In other instances, the half-life of a conjugate *in vivo* may be altered by choice of cross-bridge, especially when using cleavable reagents.

The following heterobifunctional reagents are organized according to their reactivities. The majority are commercially available and their properties well documented in the literature.

1. Amine-Reactive and Sulfhydryl-Reactive Cross-linkers

Perhaps the most popular heterobifunctional reagents are those that contain amine-reactive and sulfhydryl-reactive ends. The amine-reactive group is usually an active ester, most often an NHS ester, while the sulfhydryl-reactive portion may be one of several different functional groups. The amine-reactive end of these cross-linkers is

typically an acylating agent possessing a good leaving group that can undergo nucleophilic substitution to form an amide bond with primary amines. The sulfhydryl-reactive portion, by contrast, is usually an alkylating agent that is capable of creating either thioether or disulfide linkages with sulfhydryl-containing molecules. Depending on the chemistry chosen, linkages with a sulfhydryl-containing molecule may be either permanent covalent bonds or reversible disulfide bonds that can be cleaved by use of a suitable disulfide reductant.

The active ester chemistry of the amine-reactive end of these cross-linkers is characteristically the most labile functional group, being susceptible to rapid hydrolysis under the aqueous conditions of a conjugation reaction. The sulfhydryl-reactive group, however, is usually much more stable to breakdown in aqueous environments. Therefore, these reagents typically are used in multistep conjugation protocols wherein one protein or molecule is first modified through its amines to yield a sulfhydryl-reactive intermediate. After removal of excess cross-linker by gel filtration, a second protein or molecule containing a sulfhydryl group is added to effect the final conjugation. The stability of the sulfhydryl-reactive end of these cross-linkers allows greater control over the cross-linking process than is possible with single-step procedures.

1.1. SPDP, LC-SPDP, and Sulfo-LC-SPDP

N-Succinimidyl 3-(2-pyridyldithio)propionate (SPDP) may be the most popular heterobifunctional cross-linking agent available. The activated NHS ester end of SPDP

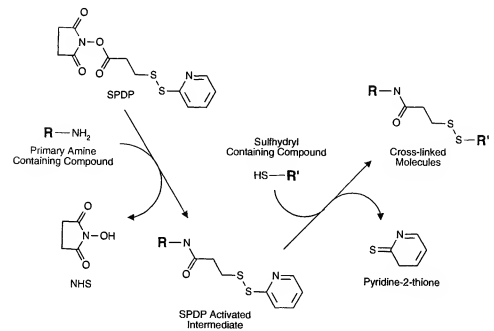
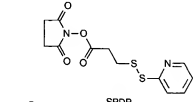
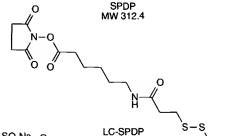


Figure 156 SPDP can react with amine-containing molecules through its NHS ester end to form amide bonds. The pyridyl disulfide group then can be coupled to a sulfhydryl-containing molecule to create a cleavable disulfide bond.

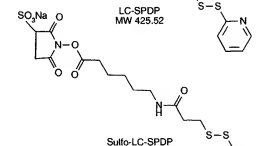
reacts with amine groups in proteins and other molecules to form an amide linkage. The 2-pyridyldithiol group at the other end reacts with sulfhydryl residues to form a disulfide linkage with sulfhydryl-containing molecules (Carlsson *et al.*, 1978) (Fig. 156). The cross-linker is used extensively to form enzyme conjugates for use in immunoassays or in labeled DNA probe techniques. It also is frequently used for the preparation of immunotoxin conjugates for *in vivo* administration (Chapter 17, Section 2.4, and Chapter 11, Section 2.1). In addition, the reagent is effective in creating sulfhydryls on proteins and other molecules (Chapter 1, Section 4.1). Once modified with SPDP, a protein can be treated with DTT (or another disulfide-reducing agent) to release the pyridine-2-thione leaving group and form the free sulfhydryl. The terminal —SH group then can be used to conjugate with any cross-linking agents containing sulfhydryl-reactive groups, such as maleimide or iodoacetyl (for covalent conjugation) or 2-pyridyldithiol groups (for reversible conjugation).



SPDP
MW 312.4



LC-SPDP
MW 425.52



Sulfo-LC-SPDP
MW 527.56

There are three forms of SPDP analogs currently commercially available (Pierce): the standard SPDP, a long-chain version designated LC-SPDP, and a water-soluble, sulfo-NHS form also containing an extended chain, called Sulfo-LC-SPDP. Both the standard SPDP and the LC-SPDP are insoluble in aqueous solutions and must be first solubilized in DMSO prior to addition to the reaction solution. The Sulfo-LC-SPDP may be solubilized directly in water or buffer. The long-chain versions extend the length of the cross-linker for those applications that require greater accessibility to react with sterically hindered functional groups. Since many sulfhydryl residues are found below the surface of a protein structure in more hydrophobic domains, the

longer spacer arm of the LC versions may be more effective in conjugations with these groups.

The following procedure is a suggested multistep protocol involving the activation of one protein by modification of its amines through the NHS ester end of SPDP, purification of this active intermediate, and subsequent addition of a sulfhydryl-containing molecule for conjugation via the remaining pyridyl disulfide group.

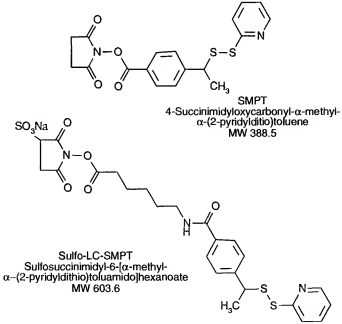
Protocol

1. Dissolve a protein or macromolecule containing primary amines at a concentration of 10 mg/ml in 50 m*M* sodium phosphate, 0.15 *M* NaCl, pH 7.2. Other non-amine-containing buffers such as borate, Hepes, and bicarbonate also may be used in this reaction. Avoid sulfhydryl-containing components in the reaction mixture, as these will react with the pyridyl disulfide end of SPDP. The effective pH for the NHS ester modification reaction is in the range of 7 to 9.
2. Dissolve SPDP at a concentration of 6.2 mg/ml in DMSO (makes a 20 m*M* stock solution). Alternatively, LC-SPDP may be used and dissolved at a concentration of 8.5 mg/ml in DMSO (also makes a 20 m*M* solution). If the water-soluble Sulfo-LC-SPDP is used, a stock solution in water may be prepared just prior to addition of an aliquot to the thiolation reaction. In this case, prepare a 10 m*M* solution of Sulfo-LC-SPDP by dissolving 5.2 mg/ml in water. Since an aqueous solution of the cross-linker will degrade by hydrolysis of the sulfo-NHS ester, it should be used quickly to prevent significant loss of activity. If a sufficiently large amount of protein will be modified, the solid may be added directly to the reaction mixture without preparing a stock solution in water to allow accurate weighing of Sulfo-LC-SPDP.
3. Add 25 µl of the stock solution of either SPDP or LC-SPDP in DMSO to each milliliter of the protein to be modified. If Sulfo-LC-SPDP is used, add 50 µl of the stock solution in water to each milliliter of protein solution.
4. Mix and react for at least 30 min at room temperature. Longer reaction times, even overnight, will not adversely affect the modification.
5. Purify the modified protein from reaction by-products by dialysis or gel filtration using 50 m*M* sodium phosphate, 0.15 *M* NaCl, 10 m*M* EDTA, pH 7.2.
6. Add a sulfhydryl-containing protein or other molecule to the purified SPDP-modified protein to effect the conjugation reaction. Molecules lacking available sulfhydryl groups may be modified to contain them by a number of methods (Chapter 1, Section 4.1). The amount of this second protein added to the reaction should be governed by the desired molar ratio of the two proteins in the final conjugate. The conjugation reaction should be done in the presence of at least 10 m*M* EDTA to prevent metal-catalyzed sulfhydryl oxidation.

1.2. SMPT and Sulfo-LC-SMPT

Succinimidyloxycarbonyl-α-methyl-α-(2-pyridyldithio)toluene (SMPT) is a heterobifunctional cross-linking agent that contains an amine-reactive NHS ester on one end and a sulfhydryl-reactive pyridyl disulfide group on the other. SMPT is therefore an analog of SPDP that differs only in its cross-bridge which contains an aromatic ring

and a hindered disulfide group (Thorpe *et al.*, 1987; Ghetie *et al.*, 1990a,b). The spacer arm of SMPT is slightly longer than SPDP (11.2 vs 6.8 Å), but the presence of the benzene ring and α-methyl group adjacent to the disulfide sterically hinders the structure sufficiently to provide increased half-life of conjugates *in vivo*.



Conjugation reactions done using SMPT often proceed by a multistep protocol involving modification of one protein through its amine groups to create a pyridyl disulfide-activated intermediate. Since SMPT is not soluble in water, the reagent is first solubilized in DMF or DMSO and an aliquot of this stock solution added to the reaction. The NHS ester end of the reagent reacts with ε- and N-terminal amine groups to create stable amide linkages. After removal of excess cross-linker by gel filtration or dialysis, a second protein containing a sulfhydryl group is added to effect the conjugation (Fig. 157). The resultant protein–protein cross-link contains a disulfide bond that is susceptible to cleavage by reduction, although more slowly due to the hindered nature of the cross-bridge.

SMPT often is used for the preparation of immunotoxin conjugates that contain a monoclonal antibody directed against some cell-surface antigen (usually a tumor-associated antigen) cross-linked to a protein toxin molecule. In has been shown that a cleavable linkage between the antibody and toxin molecules helps to ensure a potent immunotoxin (Lambert *et al.*, 1985). Increased cytotoxicity is typically observed for immunotoxin conjugates containing cross-bridge disulfides as opposed to noncleavable linkages. Cleavability presumably facilitates the release of the toxin from the antibody after the conjugate has bound to the cell surface. However, the disulfide bonds formed from some cross-linkers, such as SPDP, are readily reduced and cleaved *in vivo*—often before they reach their target. The hindered disulfide of SMPT has distinct advantages in this regard. Thorpe *et al.* (1987) showed that SMPT conjugates had approximately twice the half-life *in vivo* as SPDP conjugates.

A water-soluble analog of SMPT, called sulfo-LC-SMPT or sulfosuccinimidyl-6-

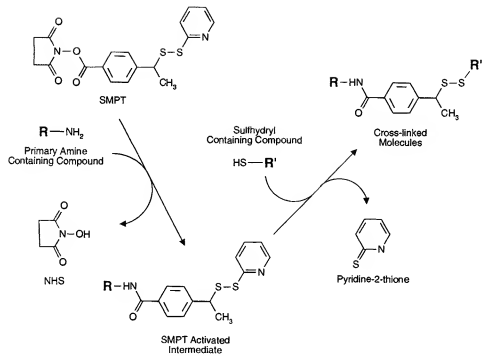


Figure 157 SMPT can form cross-links between an amine-containing molecule and a sulfhydryl-containing compound through amide and disulfide linkages, respectively. The hindered nature of the disulfide group provides better stability toward reduction and cleavage.

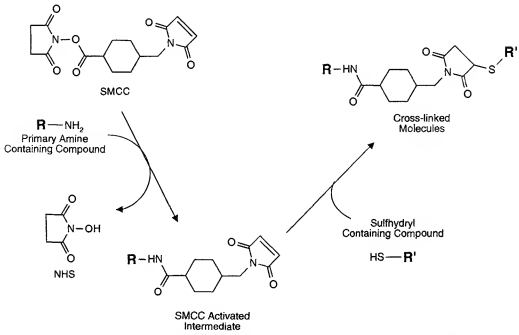
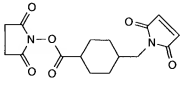


Figure 158 SMCC reacts with amine-containing molecules to form stable amide bonds. Its maleimide end then may be conjugated to a sulfhydryl-containing compound to create a thioether linkage.

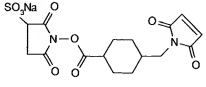
[α-methyl-α-(2-pyridyldithio)toluamido]hexanoate, is available from Pierce Chemical. The sulfo-NHS ester end of the reagent provides the water solubility due to the negative charge of the sulfonate group. Although sulfo-LC-SMPT contains the same chemical reactivity as SMPT, its cross-bridge contains an additional 6-aminocaproic acid spacer providing a 20-Å cross-link as opposed to the 11.2-Å length of SMPT. The reactivity and use of sulfo-LC-SMPT is essentially the same as that of SMPT, except that the reagent may be added directly to aqueous reaction media or predissolved in water. A stock solution made in water should be used immediately to prevent extensive NHS ester hydrolysis.

1.3. SMCC and Sulfo-SMCC

Succinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (SMCC) is a heterobifunctional cross-linker with significant utility in cross-linking proteins, particularly in the preparation of antibody–enzyme and hapten–carrier conjugates (Hashida and Ishikawa, 1985; Dewey *et al.*, 1987). The NHS ester end of the reagent can react with primary amine groups on proteins to form stable amide bonds. The maleimide end of SMCC is specific for coupling to sulfhydryls when the reaction pH is in the range of 6.5–7.5 (Smyth *et al.*, 1964) (Fig. 158).



Sulfo-SMCC;
Succinimidyl 4-(N-maleimidomethyl)-
cyclohexane-1-carboxylate
MW 334.33
11.6 Å



Sulfo-SMCC;
Sulfosuccinimidyl 4-(N-maleimidomethyl)-
cyclohexane-1-carboxylate
Water Soluble
MW 436.37
11.6 Å

At pH 7 the reaction of the maleimide group with sulfhydryls proceeds at a rate 1000 times greater than its reaction with amines. At more alkaline pH values, however, its reaction with amines becomes more evident. The maleimide end also may undergo hydrolysis to an open maleamic acid form that is unreactive toward sulfhydryls. Hydrolysis may occur after sulfhydryl coupling to the maleimide, as well. This ring-opening reaction typically happens faster the higher the pH becomes. However, the maleimide group of SMCC displays unusual stability up to pH 7.5 The increased stability of SMCC's maleimide group may be due to it not being attached directly to an

aromatic ring structure. By contrast, some maleimide-containing reagents, such as *N,N'*-o-phenylenedimaleimide and *N,N'*-oxydimethylenedimaleimide are less stable under these conditions. Reportedly, only 4% of the maleimide groups of SMCC will decompose at neutral pH within 2 h at 30°C (Ishikawa *et al.*, 1983a,b). For this reason, proteins may be modified with SMCC to form relatively long-lived, maleimide-activated intermediates. The SMCC derivative then may be freeze-dried to provide a stock preparation of sulfhydryl-reactive protein.

SMCC frequently is used to effect hapten–carrier or antibody–enzyme conjugations. In both applications, one of the molecules is activated (usually the carrier or the enzyme) with the cross-linker, purified to remove excess reagents, and then mixed with the sulfhydryl-containing second molecule to effect the conjugation. Published applications using SMCC are numerous, but include conjugation of glucose oxidase to rabbit antibodies (Yoshitake *et al.*, 1979), cross-linking Fab' fragments to horseradish peroxidase (Ishikawa *et al.*, 1983a,b; Yoshitake *et al.*, 1982a,b; Imagawa *et al.*, 1982; Uto *et al.*, 1991), coupling anti-digoxin $F(ab')_2$ fragments to β-galactosidase (Freytag *et al.*, 1984a,b), preparing conjugates of alkaline phosphatase and human IgG $F(ab')_2$ fragments (Mahan *et al.*, 1987), and use in the preparation of immunogens (Peeters *et al.*, 1989).

Since SMCC is a water-insoluble cross-linker, it must be dissolved first in organic solvent (DMF) before it is added to a protein to be modified. In some cases, addition of even a small amount of organic solvent to a protein solution may be detrimental to activity. To be safe, no more than 10–20% solvent should be present in the aqueous reaction medium.

Sulfosuccinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (sulfo-SMCC) is a water-soluble analog of SMCC that possesses a negatively charged sulfonate group on it *N*-hydroxysuccinimide ring. The charge gives just enough polarity to the molecule to provide water solubility at a level of at least 10 mg/ml at room temperature. This allows direct addition of the reagent to reaction mixtures without prior dissolution in organic solvent. The cross-linker is known to be soluble at a concentration of at least 10 m*M* in the following buffers: *(a)* 50 m*M* sodium acetate, pH 5; *(b)* 50 m*M* sodium borate, pH 7.6; *(c)* 0.1 *M* sodium phosphate, pH 6–7.5. Aqueous stock solutions also may be prepared using sulfo-SMCC, but these should be dissolved rapidly and used immediately to prevent extensive loss of sulfo-NHS coupling ability due to hydrolysis. Concentrated aqueous stock solutions (up to about 50 mg/ml) may be made by heating for a few minutes under hot running water. Quickly cool to room temperature before using.

The following is a generalized protocol for the activation of a protein with sulfo-SMCC with subsequent conjugation to a sulfhydryl-containing second molecule or protein. Specific examples of the use of this cross-linker to make antibody–enzyme or hapten–carrier conjugates may be found in Chapter 10, Section 1.1, and Chapter 9, Section 5, respectively.

Protocol

1. Dissolve 10 mg of a protein or other macromolecule to be activated with sulfo-SMCC in 1 ml of 0.1 *M* sodium phosphate, 0.15 *M* NaCl, pH 7.2.
2. Weigh out 2 mg of sulfo-SMCC and add it to the above solution. Mix gently to dissolve. To aid in measuring the exact quantity of cross-linker, a concentrated

stock solution may be made in water and an aliquot equal to 2 mg transferred to the reaction solution. If a stock solution is made, it should be dissolved rapidly and used immediately to prevent extensive hydrolysis of the active ester. As a general guideline of addition for a particular protein activation,the use of a 40- to 80-fold molar excess of cross-linker over the amount of protein present usually results in good activation levels.

3. React for 1 h at room temperature with periodic mixing.
4. Immediately purify the maleimide-activated protein by applying the reaction mixture to a desalting column packed with Sephadex G-25 or the equivalent. Do not dialyze the solution, since the maleimide activity will be lost over the time course required to complete the operation. To obtain good separation between the protein peak (eluting first) and the peak representing excess reagent and reaction by-products (eluting second), the applied sample size should be no more than 8% of the column bed volume. If complete separation of the activated protein from excess cross-linker is not obtained, then the maleimide content contributed from contaminating cross-linker may prevent subsequent conjugate formation. Perform the chromatography using 0.1 *M* sodium phosphate, 0.15 *M* NaCl, pH 7.2. Collect 1-ml fractions and pool the peak containing the protein. At this point, the maleimide-activated protein may be used immediately in a conjugation reaction with a sulfhydryl-containing protein or other molecule or freeze-dried to preserve the maleimide activity.
5. To effect the conjugation reaction, mix the maleimide-activated protein at the desired molar ratio with a sulfhydryl-containing molecule dissolved in 0.1 *M* sodium phosphate, 0.15 *M* NaCl, pH 7.2. The purified protein from step 4 may be concentrated if necessary using centrifugal concentrators, but this should be done quickly to avoid extensive loss of activity. The molar ratio of addition depends on the desired conjugate to be obtained. For instance, if coupling a sulfhydryl-containing small molecule to a protein, the molecule should be added in excess to the amount of maleimide activity present on the protein. In such a case, a 10- to 100-fold molar excess may be appropriate (Chapter 9, Section 5). However, if preparing protein–protein conjugates, as in the case of antibody–enzyme conjugates, the ratio of maleimide-activated protein to the sulfhydryl-containing protein is a matter of choice. Often, when coupling enzymes to antibodies, the enzyme is in molar excess over the antibody (see Chapter 10, Section 1.1). Typical molar ratios of enzyme-to-antibody can range from 2:1 to 7:1.
6. React for 2–24 h at room temperature or 4–24 h at 4°C.
7. The conjugate may be isolated by gel filtration if the molecular weight of the complex is sufficiently different from that of the unconjugated molecules.

1.4. MBS and Sulfo-MBS

m-Maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS) is a heterobifunctional cross-linking agent containing an NHS ester on one end and a maleimide group on the other. The NHS ester can react with primary amines in proteins and other molecules to form stable amide bonds, while the maleimide end nearly exclusively reacts with sulfhydryl groups to create stable thioether linkages (Fig. 159). These characteristics

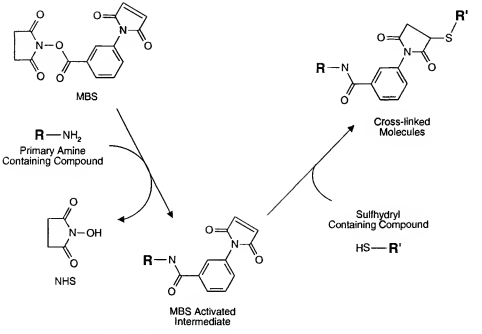
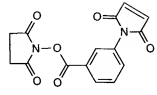


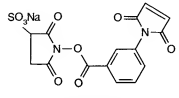
Figure 159 The two-step conjugation procedure for the MBS cross-linking of an amine-containing molecule with a sulfhydryl-containing molecule.

allow highly controlled conjugation reactions to be done with MBS using two- or three-step processes. In this sense, the NHS ester end of the reagent typically is reacted with the first protein to be cross-linked, forming a maleimide-activated intermediate. The maleimide group is more stable to breakdown by hydrolysis than the NHS ester, so the activated intermediate can be quickly purified from excess cross-linker and reaction by-products before it is added to the sulfhydryl-containing second molecule. However, due to the aromatic ring adjacent to its maleimide functional group, MBS displays less stability toward maleimide ring opening than SMCC (see this chapter Section 1.3). Unlike SMCC, MBS is therefore not recommended for preparing freeze-dried, maleimide-activated proteins, since during the processing necessary to purify and stabilize the derivative much activity can be lost by hydrolysis.

MBS contains a benzoic acid derivative as its cross-bridge, thus lending considerable hydrophobicity to the entire molecule. Because the reagent is water-insoluble, it must be first dissolved in organic solvent before it is added to an aqueous reaction medium. Making a concentrated stock solution of MBS in DMF of DMSO allows transfer of a small amount to a conjugation reaction (total concentration of the organic solvent should not exceed 10% in the reaction buffer). When these solvents are used, a microemulsion is formed in the aqueous solution, which provides cross-linker efficiently to the conjugating species. The reagent also is readily permeable to membrane structures due to its hydrophobic nature.



MBS;
m-Maleimidobenzoyl-N-hydroxy-
succinimide ester
MW 314.2
9.9 Å



Sulfo-MBS;
m-Maleimidobenzoyl-N-hydroxy-
sulfosuccinimide ester
MW 416.24
9.9 Å

m-Maleimidobenzoyl-*N*-hydroxysulfosuccinimide ester (sulfo-MBS) is a water soluble analog of MBS that contains a negatively charged sulfonate group on its NHS ring (Aithal *et al.*, 1988; Martin and Papahadjopoulos, 1982). The negative charge lends enough hydrophilicity to the cross-linker to allow direct addition of the reagent to aqueous reaction media without prior dissolution in organic solvents. Sulfo-MBS has reactivity identical to of MBS.

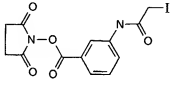
MBS was one of the first and most popular of the family of NHS ester–maleimide heterobifunctionals (Kitagawa and Aikawa, 1976). It has been used extensively to produce antibody–enzyme and other enzyme conjugates (O'Sullivan *et al.*, 1979; Freytag *et al.*, 1984; Kitagawa *et al.*, 1978), in the preparation of hapten–carrier immunogens (Liu *et al.*, 1979; Lerner *et al.*, 1981; Chamberlain *et al.*, 1989; Edwards *et al.*, 1989; Miller *et al.*, 1989; Swanson *et al.*, 1991; Kitagawa *et al.*, 1982; Niman *et al.*, 1985), and for making immunotoxin conjugates (Youle and Nevelle, 1980; Myers *et al.*, 1989; Dell'Arciprete *et al.*, 1988).

The generalized protocol for performing a multistep conjugation reaction with MBS or sulfo-MBS is similar to that described for SMCC (this chapter, Section 1.3). Specific examples may be found in the cited references.

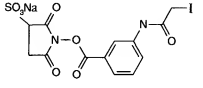
1.5. SIAB and Sulfo-SIAB

N-Succinimidyl(4-iodoacetyl)aminobenzoate (SIAB) is a heterobifunctional cross-linker containing amine-reactive and sulfhydryl-reactive ends (Weltman *et al.*, 1983). The NHS ester of SIAB can couple to primary amine-containing molecules, forming stable amide linkages (Chapter 2, Section 1.4). The other end contains an iodoacetyl group that is specific for coupling to sulfhydryl residues, creating stable thioether

bonds (Chapter 2, Section 2.1). The aminobenzoate cross-bridge is a hydrophobic spacer that helps the reagent become fully permeable to membrane structures.



SIAB;
N-Succinimidyl(4-iodoacetyl)-
aminobenzoate
MW 402.15
10.6 Å



Sulfo-SIAB;
Sulfo-succinimidyl(4-iodoacetyl)-
aminobenzoate
MW 504.2
10.6 Å

As SIAB is water-insoluble, it must be first dissolved in organic solvent prior to addition to an aqueous reaction medium. The most commonly used solvents for this purpose include DMSO and DMF. Typically, a concentrated shock solution is prepared in one of these solvents and an aliquot added to the protein conjugation solution. Long-term storage of the reagent in these solvents is not recommended, however, due to slow uptake of water and breakdown of the NHS ester end.

Conjugations done with SIAB usually proceed by a multistep process. Because the cross-linker's NHS ester end is its most labile functional group, an amine-containing protein or molecule is reacted first to create an iodoacetyl-activated intermediate (Fig. 160). This iodoacetyl derivative is stable enough in aqueous solution to allow purification of the derivatized protein from excess reagent and other reaction by-products without significant loss of activity. The only consideration is to protect the iodoacetyl derivative from light, which may generate iodine and reduce the activity of the intermediate. Finally, the modified protein is mixed with a sulfhydryl-containing molecule to effect the conjugation through a thioether bond. The result of such two-step procedures is to direct the coupling toward only sulfhydryls on the second molecule while avoiding the polymerization that can occur with single-step protocols. Conjugations done with SIAB should avoid buffer components containing amines (i.e., Tris, glycine, or imidazole) or sulfhydryls (i.e., DTT, 2-mercaptoethanol, cysteine), since these will compete with the desired cross-linking reaction.

Sulfosuccinimidyl(4-iodoacetyl)aminobenzoate (sulfo-SIAB) is a water-soluble analog of SIAB that contains a negatively charged sulfonate on its NHS ring. The negative charge lends enough hydrophilicity to the entire molecule to provide good solubility in aqueous solutions (up to about 10 m*M*). Sulfo-SIAB may be added directly to reaction mediums without prior dissolution in organic solvent, or more concentrated solutions may be made in water before transfer of an aliquot to the reaction to facilitate easy

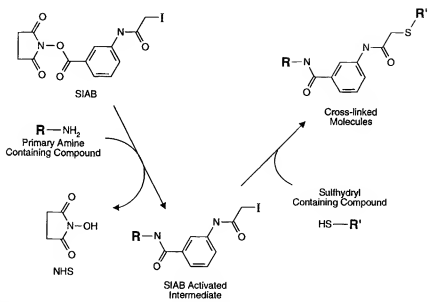


Figure 160 SIAB may be used to modify an amine-containing molecule for subsequent conjugation to a sulfhydryl-containing molecule.

addition of small quantities. Aqueous stock solutions should be dissolved rapidly and used immediately to avoid excessive hydrolysis of the NHS ester.

The following protocol illustrates the use of SIAB in preparing antibody–enzyme conjugates using β-galactosidase.

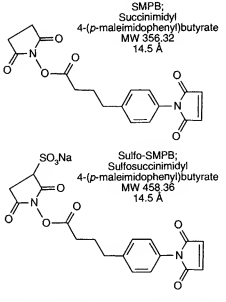
Protocol

1. Dissolve a specific antibody to be conjugated at a concentration of 10 mg/ml in 50 m*M* sodium borate, 5 m*M* EDTA, pH 8.3 (reaction buffer).
2. Dissolve SIAB (Pierce) in DMSO at a concentration of 1.4 mg/ml. Alternatively, dissolve sulfo-SIAB in deionized water at a concentration of 1.7 mg/ml. Prepare fresh and use immediately. Protect from light.
3. Add 100 µl of the SIAB stock solution to each milliliter of the antibody solution. Mix gently to dissolve.
4. React for 1 h at room temperature in the dark.
5. Purify the modified antibody by gel filtration on a Sephadex G-25 column. Perform the chromatography using the reaction buffer. To obtain good separation, apply sample at a ratio of no more than 8% of the total column gel volume. Monitor the eluting peak by using a small aliquot of each fraction and reacting it with a protein detection reagent such as Coomassie protein assay reagent (Pierce) in a microplate. This avoids exposure of the entire modified protein fractions to UV light from a spectrophotometer. Collect the first peak eluting from the column, which contains the protein.
6. Add β-galactosidase to the activated antibody solution at a ratio of 4 mg of enzyme per milligram of antibody.

7. React for 1 h at room temperature in the dark.
8. To block any remaining iodoacetyl sites, add cysteine to a final concentration of 5 m*M* and react for an additional 15 min at room temperature.
9. Purify the conjugate by gel filtration using a buffer of choice (i.e., PBS, pH 7.4).

1.6. SMPB and Sulfo-SMPB

Succinimidyl-4-(*p*-maleimidophenyl)butyrate (SMPB) is a heterobifunctional analog of MBS (this chapter, Section 1.4) containing an extended cross-bridge (Pierce). The reagent has an amine-reactive NHS ester on one end and a sulfhydryl-reactive maleimide group on the other end (Fig. 161). Conjugates formed using SMPB thus are linked by stable amide and thioether bonds. A comparison with SPDP produced conjugates concluded that SMPB formed more stable complexes that survive *in vivo* for longer periods (Martin and Papahadjopoulos, 1982).



Conjugation reactions done with SMPB typically are multistep procedures, wherein a protein is modified through its amine groups, purified to remove excess reagent, and then mixed with a sulfhydryl-containing molecule to effect the final conjugation. The maleimide group of SMPB is highly specific for coupling to sulfhydryl-containing proteins and other molecules, thus directing the conjugation to discrete points on the second molecule. This maleimide is, however, more labile to ring opening in aqueous solution than the maleimide group of SMCC due to its proximity to an aromatic ring. Therefore, the first protein modified with SMPB (to obtain a maleimide-activated intermediate) should be purified quickly to prevent extensive activity loss from hydrolysis and maleimide ring opening.

SMPB contains a hydrophobic cross-bridge and relatively nonpolar ends, which allows the reagent to permeate membrane structures. Due to its water-insolubility, it must be dissolved in an organic solvent prior to addition of an aliquot to a reaction mixture. The solvents DMF and DMSO work well for this purpose. A concentrated

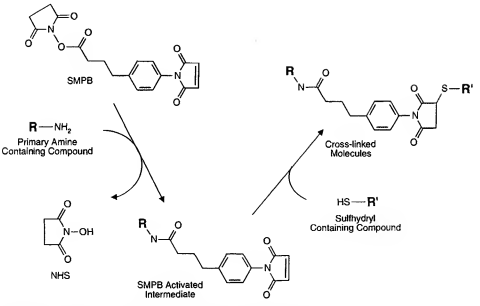


Figure 161 SMPB may be used in a two-step procedure to conjugate an amine-containing molecule to a sulfhydryl compound, forming amide and thioether bonds, respectively.

stock solution prepared in these solvents allows easy addition of a small amount to a conjugation reaction. Long-term storage in these solvents is not recommended due to slow water pickup and possible hydrolysis of the NHS ester end.

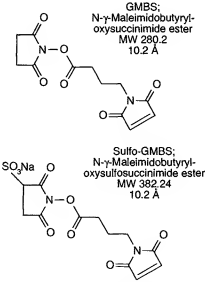
A water-soluble analog to SMPB, sulfosuccinimidyl-4-(*p*-maleimidophenyl)butyrate (sulfo-SMPB), contains a negatively charged sulfonate group that lends considerable hydrophilicity to the molecule (Pierce). Sulfo-SMPB may be added directly to aqueous reaction mixtures without prior dissolution in organic solvent. Concentrated stock solutions made in water should be dissolved quickly and used immediately to prevent hydrolysis of the NHS ester.

SMPB or sulfo-SMPB have been used to conjugate preformed vesicles and Fab' fragments in a liposome carrier study (Martin and Papahodjopoulos, 1982), to attach insulin molecules to reconstituted Sendai virus envelopes (Gitman *et al.*, 1985a,b), for targeting of loaded virus envelopes by covalently attaching insulin molecules to receptor-depleted cells (Gitman *et al.*, 1985b), in forming alkaline phosphatase-Fab' fragment conjugates for an ELISA (Teale and Kearney, 1986), in preparing peptide–protein immunogen conjugates (Iwai *et al.*, 1988), in studying the transport of the variant surface glycoprotein of Trypanosome brucia (Bangs *et al.*, 1986), and in preparing immunotoxin conjugates (Myers *et al.* 1989).

1.7. GMBS and Sulfo-GMBS

N-(γ-Maleimidobutyryloxy)succinimide ester (GMBS) is a heterobifunctional cross-linking agent that contains an NHS ester on one end and a maleimide group on the other (Fujiwara *et al.*, 1988) (Pierce). Its internal cross-bridge contains a linear 4-car-

bon spacer, resulting in a 10.2-Å cross-links between conjugated molecules (Fig. 162). GMBS is water-insoluble and therefore must be dissolved in organic solvent prior to use. Typically, a concentrated stock solution is prepared in DMF or DMSO just before use, and then an aliquot of the solution is transferred to the aqueous reaction medium. The result is the formation of a microemulsion that effectively supplies cross-linker to the aqueous phase.



GMBS can be used in multistep conjugation protocols wherein an amine-containing molecule or protein is first modified via the NHS ester end (its most labile functional group) to create a stable amide bond. The derivative at this point contains reactive

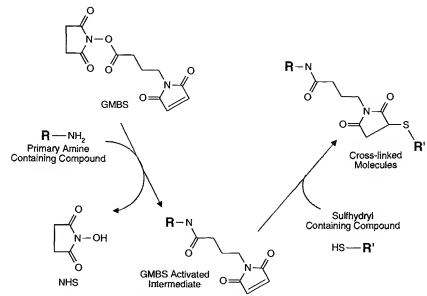


Figure 162 The reaction of GMBS with an amine-containing molecule yields a maleimide-activated intermediate that then can be used to cross-link with a sulfhydryl-containing compound.

maleimide groups able to couple with the available sulfhydryl groups of a second protein or molecule. This active intermediate then is purified to remove excess reagent and reaction by-products, and immediately added to the sulfhydryl-containing molecule to effect the final conjugation.

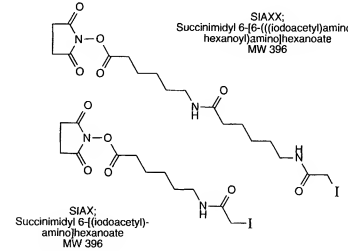
The maleimide group of GMBS is adjacent to an aliphatic spacer, so its stability toward ring opening should be better than cross-linkers like MBS, which contain adjacent aromatic groups. Hydrolysis of the maleimide group results in loss of sulfhydryl coupling capability. However, GMBS is not as stable as the hindered maleimide group of SMCC, since the cyclohexane ring of that reagent inhibits hydrolysis and ring opening.

N-(γ-Maleimidobutyryloxy)sulfosuccinimide ester (sulfo-GMBS) is a water-soluble analog of GMBS containing a negatively charged sulfonate group on its NHS ring (Pierce). The charge provides enough hydrophilicity to allow at least 10 m*M* concentrations of the cross-linker to be made in aqueous reaction mediums. Its reactivity is identical to that of GMBS.

The protocol for using GMBS or sulfo-GMBS in protein–protein cross-linking applications is similar to that of SMCC or sulfo-SMCC (see Section 1.3).

1.8. SIAX and SIAXX

Succinimidyl 6-((iodoacetyl)amino)hexanoate (SIAX) is a heterobifunctional reagent containing an NHS ester on one end and an iodoacetyl group on the other (Brinkley, 1992) (Molecular Probes). The NHS ester reacts with primary amines in proteins and other molecules to form stable amide bonds. The iodoacetyl group is highly specific for sulfhydryl groups, reacting to create stable thioether linkages (Fig. 163). The reactivity and use of this cross-linker is similar to that of SIAB, described previously. SIAX possesses a 6-aminohexanoic acid internal cross-bridge, providing a total of a 9-atom spacer between conjugated molecules.



SIAX is a hydrophobic reagent that should penetrate membrane structures with good efficiency. The cross-linker must be solubilized in organic solvent (DMF or DMSO) prior to transfer of a small amount to an aqueous reaction medium.

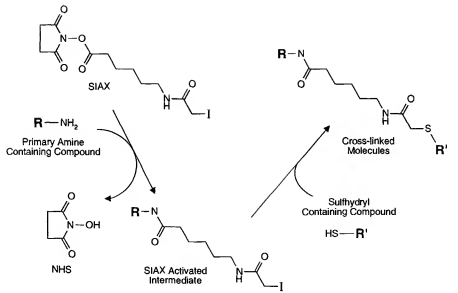


Figure 163 SIAX can be used to modify amine-containing molecules to produce sulfhydryl-reactive derivatives. Subsequent reaction with a thiol compound produces a thioether linkage.

Succinimidyl 6-(6-(((4-iodoacetyl)amino)hexanoyl)amino)hexanoate (SIAXX) is a long-chain analog of SIAX that contains two aminohexanoate spacer groups in its cross-bridge, instead of one (Molecular Probes). Conjugates prepared with this re-

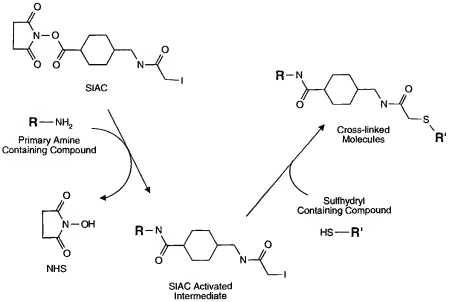


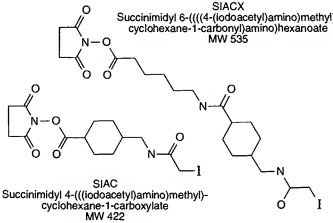
Figure 164 SIAC reacts with an amine-containing compound to yield an amide bond derivative that is reactive toward thiol-containing molecules. Secondary reaction with a sulfhydryl group gives a stable thioether bond.

agent are connected by a spacer arm containing 16 atoms. Like SIAX, SIAXX must be first solubilized in DMF or DMSO before it is added to a buffered reaction. The increased chain length SIAXX, however, does not affect its reactivity toward amines and sulfhydryls.

Conjugation reactions done with SIAX or SIAXX are usually multistep procedures similar to the protocol described previously for SIAB.

1.9. SIAC and SIACX

Succinimidyl 4-(((iodoacetyl)amino)methyl)cyclohexane-1-carboxylate (SIAC) is a heterobifunctional reagent containing an NHS ester on one end and a iodoacetyl group on the other (Molecular Probes). The cross-linker can react with amine groups via its NHS ester end to form stable amide bonds, while its iodoacetyl functional group can couple to sulfhydryl groups, creating stable thioether linkages (Fig. 164). SIAC contains a cross-bridge made from a cyclohexane derivative, which provides approximately an 8-atom spacer between conjugated species.



SIAC is a hydrophobic cross-linker that must be solubilized in organic solvent (DMF or DMSO) prior to addition of an aliquot to an aqueous reaction medium. It should exhibit good membrane permeability.

Succinimidyl 6-((((4-iodoacetyl)amino)methyl)cyclohexane-1-carbonyl)amino)-hexanoate (SIACX) is an analog of SIAC that contains an additional aminohexanoate spacer group next to its NHS ester end (Molecular Probes). The result is the creation of an approximately 16-atom spacer arm between conjugated molecules. All other properties of SIACX are similar to those of SIAC.

Conjugation reactions done with SIAC or SIACX are usually multistep procedures similar to the protocol described for SIAB, previously.

1.10. NPIA

p-Nitrophenyl iodoacetate (NPIA) is a heterobifunctional reagent based upon iodoacetate that has been activated at its carboxylic acid group with a *p*-nitrophenyl ester (Huang *et al.*, 1975; Hudson and Weber, 1973) (Molecular Probes). This active

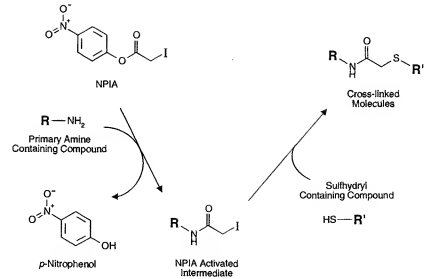
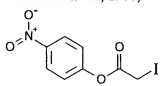


Figure 165 NPIA is one of the shortest heterobifunctional reagents. It reacts with amine-containing molecules through its *p*-nitrophenyl ester end to produce amide bonds. The iodoacetyl group then can be used to couple with thiol compounds to give stable thioether linkages.

ester species has much the same reactivity as an NHS ester, being highly reactive with amines at slightly basic pH values (pH 7–9). The *p*-nitrophenyl ester couples to amine-containing proteins and other molecules to form stable amide linkages. The other end of the short cross-linker can react with sulfhydryl groups to create thioether bonds. This is the smallest heterobifunctional iodoacetate-containing cross-linker available, forming only 2-atom cross-bridges between conjugated molecules (Fig. 165). NPIA has been used to investigate close interactions between biological molecules (Hiratsuka, 1987; Sutoh and Hiratsuka, 1988).



NPIA
p-Nitrophenyl Iodoacetate
MW 307

NPIA is water-insoluble and should be dissolved in DMF or methylene chloride prior to addition of an aliquot to an aqueous reaction medium. Conjugation reactions done with NPIA are usually multistep procedures similar to the protocol previously described for SIAB.

2. Carbonyl-Reactive and Sulfhydryl-Reactive Cross-linkers

A relatively new set of heterobifunctional cross-linking agents now are available that contain a carbonyl-reactive group on one end and a sulfhydryl-reactive functional

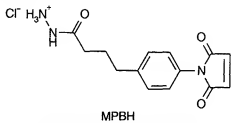
group on the other end. The main utility of these reagents is in conjugating carbohydrate-containing molecules, such as glycoproteins, to sulfhydryl-containing molecules. Both polysaccharide residues and sulfhydryl groups usually are present on proteins in limiting quantities and at discrete sites. In certain cases, conjugation through these groups can direct the coupling reaction away from critical active centers or binding sites, thus preserving activity of the proteins after cross-linking. A prime example of the advantages of this type of directed coupling can be seen when conjugating antibody molecules to other proteins, such as enzymes. The carbohydrate residues of immunoglobulin molecules usually occur on the Fc portion, away from the antigen binding sites. Coupling procedures that direct the cross-linking reaction to parts on the antibody far removed from the antigen binding sites have the best chance of retaining activity after conjugate formation.

The carbonyl-reactive functional group on these cross-linkers is a hydrazide group that can form hydrazone bonds with aldehyde residues. To utilize this functional group with carbohydrate-containing molecules, the sugars first must be mildly oxidized to contain aldehyde groups by treatment with sodium periodate. Oxidation with this compound will cleave adjacent carbon—carbon bonds that possess hydroxyl groups, as are abundant in polysaccharide molecules (Chapter 1, Sections 2 and 4.4).

Two types of sulfhydryl-reactive functions are available on these reagents: pyridyl disulfide groups and maleimide groups. The pyridyl disulfide group will react with a sulfhydryl residue to create a disulfide bond. This linkage is reversible by treatment with a disulfide reducing agent. Reaction of a maleimide group with a sulfhydryl, however, forms a permanent thioether bond of good stability. Thus, either reversible or permanent conjugates may be designed using these heterobifunctionals.

2.1. MPBH

4-(4-*N*-Maleimidophenyl)butyric acid hydrazide (MPBH) is a heterobifunctional cross-linking agent that contains a carbonyl-reactive hydrazide group on one end and a sulfhydryl-reactive maleimide on the other (Pierce). The cross-bridge between the two functional ends provides a long, 17.9-Å spacer. The hydrazide group is produced as the hydrochloride salt. The reagent as a whole has a good water solubility. It can be dissolved in 0.1 *M* sodium acetate, pH 5.5, up to a concentration of 327 mg/ml. It is also freely soluble in DMSO and may be stored as concentrated stock solution in this solvent without degradation.



MPBH
4-(4-N_Maleimidophenyl)butyric
acid hydrazide hydrochloride
MW 309.75
17.9 Å

The maleimide group of MPBH is adjacent to an aromatic ring and thus may exhibit instability to hydrolysis in aqueous solutions, especially at alkaline pH. Hydrolysis

opens the maleimide ring and destroys its coupling ability with sulfhydryls. However, both reactive ends of the cross-linker are stable enough to survive a multistep coupling protocol without extensive loss of activity. Thus, a sulfhydryl-containing protein or molecule may be modified via the maleimide end of MPBH and the derivative purified by gel filtration to remove excess reactants, and then mixed with a glycoprotein (that had been previously oxidized to provide aldehyde residues) to effect the final conjugation (Fig. 166). The opposite approach also is possible: modification of the glycoprotein first, purification, and subsequent mixing with a sulfhydryl-containing molecule. With this second option, however, the purification step should be done quickly to prevent extensive hydrolysis of the maleimide group.

MPBH has been used to conjugate CD4 without loss of biological activity (Chamow *et al.*, 1992).

2.2. M_2C_2H

4-(*N*-Maleimidomethyl)cyclohexane-1-carboxyl-hydrazide (M_2C_2H) is a heterobifunctional cross-linking agent that contains a carbonyl-reactive hydrazide group on one end and a sulfhydryl-reactive maleimide group on the other (Pierce). The reagent is similar to MPBH (described previously), but the maleimide group on M_2C_2H is expected to be more stable in aqueous solutions, since it is adjacent to an aliphatic cyclohexane ring instead of an aromatic phenyl group. In this sense, the cross-bridge of M_2C_2H is nearly identical to that of SMCC, which contains one of the most stable maleimide groups known. The hindered environment of the cyclohexane ring should

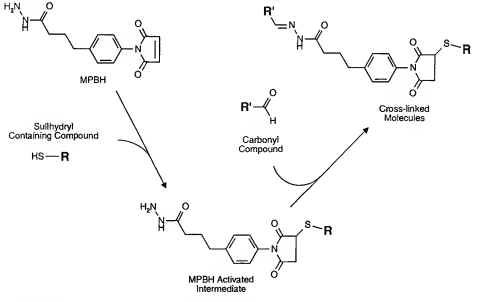
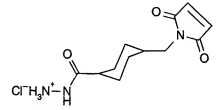


Figure 166 MPBH reacts with sulfhydryl-containing molecules through its maleimide end to produce thioether linkages. Its hydrazide group then can be used to conjugate with carbonyl-containing molecules (such as periodate oxidized carbohydrates which contain aldehydes) to give hydrazone bonds.

provide similar stability advantages to this reagent. Reaction of the maleimide group with a sulfhydryl residue results in the formation of a stable thioether bond.



M_2C_2H
4-(N-Maleimidomethyl)cyclohexane-
1-carboxyl-hydrazide hydrochloride
MW 287.75
15.1 Å

On the other end of the cross-linker, the hydrazide functional group can react with periodate-oxidized carbohydrate molecules to form hydrazone linkages (Chapter 1, Sections 2 and 4.5). Thus, glycoproteins can be targeted specifically at their polysaccharide chains, avoiding cross-linking at active sites which can lead to activity losses (Fig. 167).

M_2C_2H is slightly soluble in aqueous solutions, reportedly having a maximal solubility of 3.2 mg/ml in 0.1 *M* sodium acetate at pH 5.5. It is also soluble in organic solvents, which allows for the preparation of concentrated stock solutions to be made

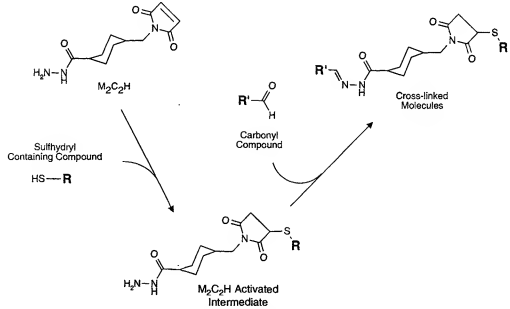
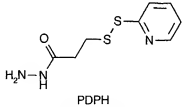


Figure 167 M_2C_2H can be used to cross-link a sulfhydryl-containing molecule with an aldehyde-containing compound. Glycoproteins may be conjugated using this reagent after treatment with sodium periodate to form reactive aldehyde groups.

prior to addition of a small aliquot to an aqueous reaction mixture. The cross-linker is particularly stable in acetonitrile.

2.3. PDPH

3-(2-Pyridyldithio)propionyl hydrazide (PDPH) is a heterobifunctional reagent that possesses a carbonyl-reactive hydrazide group on one end and a sulfhydryl-reactive pyridyl disulfide group on the other (Pierce). Thus, sulfhydryl-containing proteins or other molecules may be conjugated to carbohydrate-containing molecules (after treatment of the polysaccharide portion with sodium periodate) to create aldehyde residues (Fig. 168). Using this cross-linker, glycoproteins can be coupled specifically through their carbohydrate chains, in many cases better avoiding active centers or binding sites than when coupling through abundant polypeptide groups like amines. Since the pyridyl disulfide group reacts with sulfydryls to create disulfide bonds, the cross-linked proteins can be cleaved by reduction with DTT (Chapter 1, Section 4.1).



PDPH
3-(2-Pyridyldithio)
propionyl hydrazide
MW 229.31
9.2 Å

PDPH also may be used as a thiolation reagent to add sulfhydryl functional groups to carbohydrate molecules. The reagent can be used in this sense similar to the protocol described for AMBH (Chapter 1, Section 4.1). After modification of an oxidized polysaccharide with the hydrazide end of PDPH, the pyridyl group is removed by treatment with DTT, leaving the exposed sulfhydryl (Fig. 169).

PDPH is soluble in 0.1 *M* sodium acetate, pH 5.5, at a maximal concentration of 14.2 mg/ml. The reagent is particularly stable in acetonitrile for preparation of concentrated stock solutions.

PDPH has been used in the preparation of immunotoxin conjugates (Zara *et al.*, 1991). It has also been used to create a unique conjugate of NGF with an antibody directed against the transferrin receptor OX-26, which could traverse the blood–brain barrier (Friden *et al.*, 1993). Labeling of antibody molecules with PDPH at oxidized polysaccharide sites followed by reduction to free the sulfhydryl has been used to form a technetium-99m complex for radiopharmaceutical use (Ranadive *et al.*, 1993) (Chapter 8, Section 2.5).

3. Amine-Reactive and Photoreactive Cross-linkers

An important class of heterobifunctional reagents is the photoreactive cross-linkers that have one end that can be photolyzed to initiate coupling. Photoreactive cross-

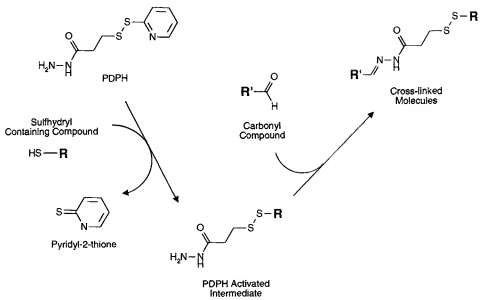


Figure 168 PDPH reacts with thiol-containing compounds through its pyridyl disulfide end to form reversible disulfide linkages. Its hydrazide end then may be subsequently conjugated with an aldehyde-containing molecule to form hydrazone bonds. Glycoproteins may be cross-linked using this approach after periodate activation to generate formyl groups.

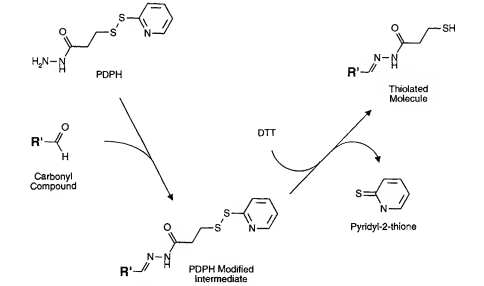


Figure 169 PDPH may be used to add a sulfhydryl group to an aldehyde-containing molecule. After reacting its hydrazide end with the aldehyde to form a hydrazone bond, the pyridyl disulfide may be reduced with DTT to create a free thiol.

linkers may be designed to utilize any one of a number of photosensitive groups, including aryl azides, fluorinated aryl azides, benzophenones, certain diazo compounds, and diazirine derivatives (Chapter 2, Section 7). The best photoreactive functional groups are stable in aqueous solution in the dark, and may be activated at the desired time by a pulse of light at the appropriate wavelength. The other end of these heterobifunctionals usually contains a spontaneously reactive functional group that will couple rapidly with certain groups present on target molecules. This secondary functionality is sometimes called *thermoreactive* to differentiate it from the photoreactive end and to emphasize its ready-reactivity or sometimes its labile nature in aqueous environments. The thermoreactive end is typically amine-reactive, sulfhydryl-reactive, carbonyl-reactive, carboxylate-reactive, or arginine-reactive. Still another class of photoreactive heterobifunctionals may use a biotin handle at one end to cross-link specifically, but noncovalently, with avidin or streptavidin molecules (Chapter 8, section 3.4).

Photoreactive groups can be categorized by the reactive species that is generated upon photolysis. The most popular type of photosensitive functional groups, aryl azide derivatives, form short-lived nitrenes that react extremely rapidly with the surrounding chemical environment (Gilchrist and Rees, 1969). Recent evidence, however, indicates that the photolyzed intermediates of aryl azides can undergo ring expansion to create nucleophile-reactive dehydroazepines. Instead of inserting nonselectively at active carbon—hydrogen bonds, dehydroazepines have a tendency to react preferentially with nucleophiles, especially amines (Fig. 170). However, some investigators have shown that aryl azides that possess a perfluorinated ring structure or are substituted completely with halogen atoms are quite efficient at forming the desired nitrene intermediate (Soundararajan *et al.*, 1993; Keana and Cai, 1990; Schnapp and Platz, 1993; Schnapp *et al.*, 1993; Cai *et al.*, 1993; Yan *et al.*, 1994). Unfortunately, at the present time, commercially available perfluorinated aryl azides are scarce.

One advantage of aryl azide photoreactive cross-linkers is that they have a relatively low energy of activation, which is optimal in the long UV region. In addition, many aryl azides possess nitro groups on their associated aromatic ring structures. These electron-withdrawing groups tend to increase the optimal wavelength for photolysis upward close to the 350-nm range. The benefit of this approach is that relatively low light exposure at higher energy UV wavelengths avoids potential bond breakage that may occur with some sensitive compounds upon photolysis.

Other phenyl azide-containing reagents possess hydroxyl groups on their aromatic rings. These electron-donating groups activate the ring system to allow electrophilic substitution reactions to occur on the cross-linker prior to its use. A major application of this ability is to radioiodinate the photoreactive reagent for detection purposes before the modification reaction with target molecules has taken place.

Suitable light sources for photolyzing include sunlamps manufactured by a number of companies, such as Philips Ultrapnil MLU 300 W, General Electric Sunlamp RSM 275 W, or National Self-Ballasted BHRF 240-250 V 250 W-P lamp. Irradiation for 15 min with such lamps while the sample is cooled in an ice bath will result in good photolysis of photoreactive cross-linkers and modification reagents.

Although photoreactive aryl azides are relatively inert to thermochemical reactions prior to photolysis, they are not stable in the presence of sulfhydryl compounds, which can reduce the azide functional group to an amine. Avoid, therefore, the use of reductants such as DTT or 2-mercaptoethanol before the photolyzing step, as these can react with the aryl azide within minutes (Staros *et al.*, 1978). Avoid also amine-containing

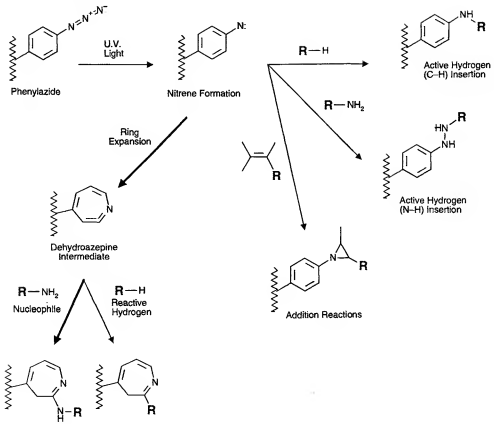


Figure 170 Photolyzing a phenyl azide group with UV light results in the formation of a short-lived nitrene. Nitrenes may undergo a number of reactions, including insertion into active carbon—hydrogen or nitrogen—hydrogen bonds and addition to points of unsaturation in carbon chains. The most likely route of reaction, however, is to ring-expand to a dehydroazepine intermediate. This group is highly reactive toward nucleophiles, especially amines.

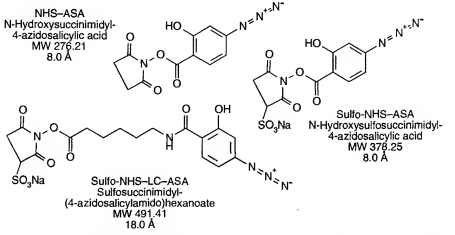
buffer components such as Tris or glycine, because of the potential for nucleophilic reactivity of the photolyzed intermediate.

Of the following amine-reactive and photoreactive cross-linkers, the overwhelming majority use an aryl azide group as the photosensitive function. Only a few use alternative photoreactive chemical reactions, particularly perfluorinated aryl azide, benzophenone, or diazo compounds. For general background information on photoreactive cross-linkers see Das and Fox (1979), Kiehm and Ji (1977), Vanin and Ji (1981), Meijer *et al.*, 1988), and Brunner (1993).

3.1. NHS-ASA, Sulfo-NHS-ASA, and Sulfo-NHS-LC-ASA

N-Hydroxysuccinimidyl-4-azidosalicylic acid (NHS-ASA) is a heterobifunctional reagent containing an NHS ester on one end and a photoreactive aryl azide group on the

other (Pierce). The amine-reactive NHS ester can be reacted with proteins or other primary amine-containing molecules to yield a photosensitive derivative suitable for probing biological interaction sites. Upon photolysis with a long UV light source, the aryl azide end is activated to complex covalently with closely associated target molecules (Fig. 171). The small cross-bridge of NHS-ASA is built from a salicylate derivative that contains a hydroxyl group on the aromatic ring. The ring-activating nature of this group provides an iodination site on the cross-linker to allow tracking of modified molecules (Ji and Ji, 1982) (Chapter 8, Section 4.5).



Reported applications of NHS-ASA include photoaffinity labeling of ^{125}I-ASA-Con A to erythrocyte ghosts (Ji and Ji, 1982), derivatization of human choriogonadotropin with ^{125}I-NHS-ASA with photo-initiated cross-linking of the α-β dimer (Ji *et al.*, 1985), radiolabeling of D-glucose and conjugation of the sugar to the human erythrocyte monosaccharide transporter protein (Shanahan *et al.*, 1985), and photoaffinity labeling of a bacterial sialidase (van der Horst *et al.*, 1990).

Two analogs of NHS-ASA that provide alternative physical characteristics are available. Sulfo-NHS-ASA is a water-soluble version of the cross-linker that contains a negatively charged sulfonate group on its NHS ring. Sulfo-NHS-LC-ASA also has the water-solubility advantage provided by a sulfonate, but possesses a longer cross-bridge made from a 6-aminocaproic acid chain in its internal structure. The longer spacer increases the potential distance between conjugated molecules, thus allowing more flexibility in the experimental design. Both analogs are still iodinatable to provide radiolabeling capability.

3.2. SASD

Sulfosuccinimidyl-2-(*p*-azidosalicylamido)ethyl-1,3′-dithiopropionate (SASD) is a heterobifunctional cross-linker containing a photoreactive group and an amine-reactive NHS ester (Pierce). The NHS ring possesses a negatively charged sulfonate group that lends water solubility to the reagent. The cross-bridge of SASD contains a central disulfide group that provides cleavability after conjugation. Reaction with a disulfide reducing agent such as DTT breaks the disulfide bond and releases the cross-

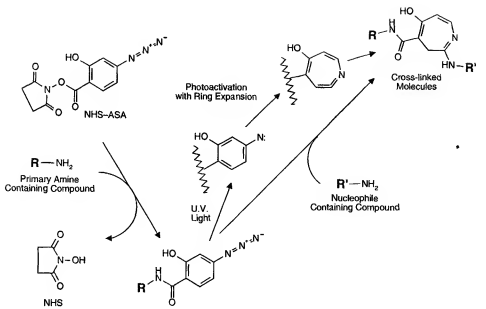
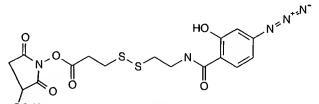


Figure 171 NHS-ASA reacts with amine-containing compounds to form stable amide linkages. Photolyzing with UV light results in ring expansion to a dehydroazepine intermediate, which can react with amines to form covalent bonds.

linked molecules. The photosensitive end of SASD is built from a salicylic acid derivative that contains a ring-activating hydroxyl group. Due to the presence of this group, the cross-linker can be radiolabeled with ^{125}I prior to a conjugation reaction. Iodination occurs *ortho* or *para* to the hydroxyl group on the phenyl ring, next to the aryl azide function (Fig. 172) (Chapter 8, Section 4.5).



SASD
Sulfosuccinimidyl-2-(*p*-azido-salicylamido)ethyl-1,3'-dithiopropionate
MW 541.51
18.9 Å

The combination of radiolabeling and cleavability provides the ability to detect the fate of the protein that retains the radiolabel after disulfide reduction. Thus, for investigations involving biomolecular interactions, a purified protein can be labeled with SASD through its amine groups via the NHS ester end of the cross-linker, allowed to interact *in vivo* with unknown target molecules, and photolyzed to effect a cross-link with these unknown substances. Subsequently the complex can be localized in the cell or effectively isolated by following the radiolabel. Alternatively, the conjugate can

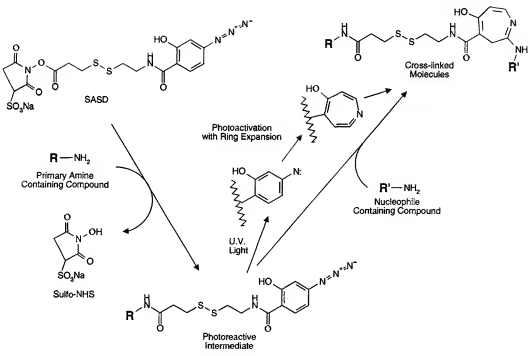


Figure 172 SASD is a photoreactive cross-linker that can be used to modify amine-containing compounds through its NHS ester end and subsequently photolyzed to initiate coupling with nucleophiles (after ring expansion to an intermediate dehydroazepine derivative). The cross-links may be selectively cleaved at the internal disulfide group using DTT.

be cleaved by reduction and the unknown molecule's fate identified through the radiolabel (Fig. 173).

Reported applications of SASD involve modification of lipopolysaccharide (LPS) molecules and studying their interaction with albumin and an antibody directed against LPS (Wollenweber and Morrison, 1985), identification of the murine interleukin-3 receptor and an *N*-formyl peptide receptor (Sorenson *et al.*, 1986), cross-linking of factor V and Va to iodinated peptides (Chattopadhyay *et al.*, 1992), and a comparison of radiolabeling techniques for the cross-linker (Shephard *et al.*, 1988).

The best radiolabeling technique for SASD is to use the IODO-GEN method (Shephard *et al.*, 1988) described in Chapter 8, Section 4.3. The following suggested protocol for using SASD was based on the method described in the Pierce Catalog.

Protocol

The following operations should be done using standard safety procedures for working with radioactive compounds. All steps involving SASD prior to initiation of the photoreaction should be done protected from light to avoid loss of phenyl azide activity. The radiolabeling procedure should be done quickly to prevent excessive loss of NHS ester activity due to hydrolysis.

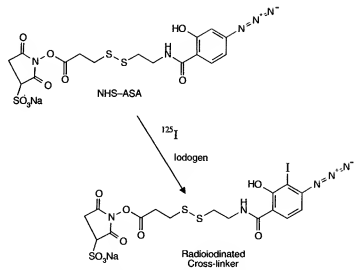


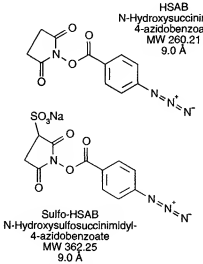
Figure 173 The hydroxyl group on the phenyl azide ring of SASD may be iodinated with ^{125}I to allow radiolabeling studies to be done on photolyzed conjugates.

1. Radiolabel 55 nmol of SASD using IODO-GEN (Pierce Chemical) and 40 μCi $Na^{125}I$ for 30 s. Do not use Chloramine-T, since termination of the iodination reaction with this reagent involves addition of a reducing agent that may cleave the disulfide bonds of the cross-linker.
2. Terminate the iodination by removing the SASD solution from the IODO-GEN reagent using a transfer pipette. Be careful not to carry any solid IODO-GEN reagent with the transfer. Since free radioactive iodine still may be present in the solution, it may be necessary to add an iodine scavenger to prevent the possibility of radiolabels being incorporated into the proteins being cross-linked. Suitable scavengers include tyrosine or *p*-hydroxyphenylacetic acid. Adding these compounds in molar excess to the amount of iodine present will prevent any secondary modifications from occurring. Immediately add the radiolabeled SASD solution to the equivalent of 16 nmol of a protein to be modified. The protein should be dissolved previously in a minimum quantity of 0.1 *M* sodium borate, pH 8.4 (conjugation buffer). The more concentrated the protein, the more efficient will be the modification reaction.
3. React for 30 min to create the SASD derivative, coupled through the NHS ester functional groups of the cross-linker onto available amine groups of the protein (forming amide bonds).
4. Purify the modified protein by desalting using a Sephadex G-25 column or the equivalent and performing the chromatography using a buffer of choice. Pool fractions containing protein. The protein should be radiolabeled at this point and also contain photoreactive phenyl azide groups from the SASD modification.
5. Add the SASD-modified protein to a second protein or other molecule to be

conjugated. After mixing, photolyze the solution with long-wave UV light for 10 min at room temperature to effect the conjugation.

3.3. HSAB and Sulfo-HSAB

N-Hydroxysuccinimidyl-4-azidobenzoate (HSAB) is a heterobifunctional reagent containing an amine-reactive NHS ester on one end and a photoreactive phenyl azide group on the other (Pierce). The small cross-bridge, built from a benzoic acid group, provides cross-linking ability at short intermolecular distances. Reaction of one protein via the NHS ester end of the cross-linker provides a stable derivative that can be incubated with a target molecule and then photolyzed to effect the final conjugation (Fig. 174).



Reactions done with HSAB should involve predissolution of the cross-linker in organic solvent prior to addition to a molecule to be modified. DMSO or DMF are suitable solvents to prepare concentrated stock solutions. Protect all solutions from light to avoid loss of photoreactive phenyl azide groups prior to the desired point of photolysis.

Reported applications of HSAB include photoaffinity labeling of peptide hormone binding sites (Galardy *et al.*, 1974), photoaffinity labeling of the insulin receptor with derivatized insulin analog (Yeung *et al.*, 1980), identifying nerve growth factor receptor proteins in sympathetic ganglia membranes (Massague *et al.*, 1981), labeling of the hormone receptor of both α and β subunits of human choriogonadotropin (Ji and Ji, 1981), isolation of *in situ* cross-linked ligand–receptor complexes (Ballmer-Hofer *et al.*, 1982), and cross-linking vasoactive intestinal polypeptide to its receptors on intact human lymphocytes (Wood and O'Dorisio, 1985).

N-Hydroxysulfosuccinimidyl-4-azidobenzoate (sulfo-HSAB) is a water-soluble analog of HSAB possessing a negatively charged sulfonate group on its NHS ring. This cross-linker may be added directly to aqueous reaction media without prior dissolution in organic solvent. To aid in the addition of small quantities of the reagent, a concentrated solution of sulfo-HSAB may be made in water and then an aliquot added

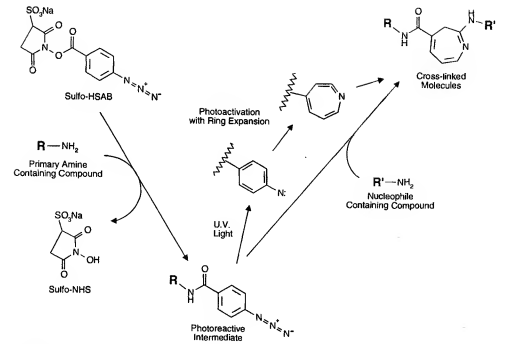
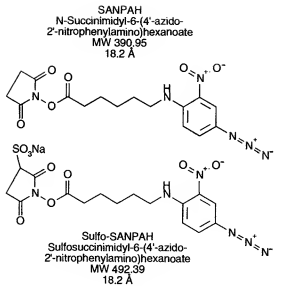


Figure 174 Sulfo-HSAB is a short photoreactive cross-linker that can be used to modify amine-containing molecules through its NHS ester end to form amide linkages. After photolysis, the phenyl azide group can react with amines to create a covalent bond.

to the reaction. Aqueous stock solutions should be dissolved quickly and used immediately to prevent extensive hydrolysis of the NHS ester.

3.4. SANPAH and Sulfo-SANPAH

N-Succinimidyl-6-(4′-azido-2′-nitrophenylamino)hexanoate (SANPAH) is a heterobifunctional cross-linking agent containing an NHS ester and a photoreactive phenyl azide group (Pierce). The NHS ester end can react with amine groups in proteins and other molecules, forming stable amide linkages. The photoreactive end is sensitive to long UV light, being selectively activated to a highly reactive nitrene or dehydroazepine intermediate. Either of these photolyzed species can couple to molecules within van der Waals contact, rapidly forming covalent bonds (Fig. 175). The cross-bridge of SANPAH is a noncleavable 6-aminohexanoic acid derivative that provides a long spacer between conjugated molecules. The phenyl azide group also contains a nitro group on the ring that has the effect of increasing the wavelength of optimal photolysis. Exposure to light at a wavelength in the range of 320–350 nm promotes the photoreaction process. SANPAH is a water-insoluble cross-linker that will permeate membrane structures efficiently. The reagent should be dissolved in DMSO or DMF prior to addition of an aliquot to an aqueous reaction medium.



Reported applications of SANPAH include the cross-linking of ligand–receptor complexes *in situ* (Ballmer-Hofer *et al.*, 1982), preparing photoactivatable glycopeptide derivatives for site-specific labeling of lectins (Baenziger and Fiete, 1982), photo-

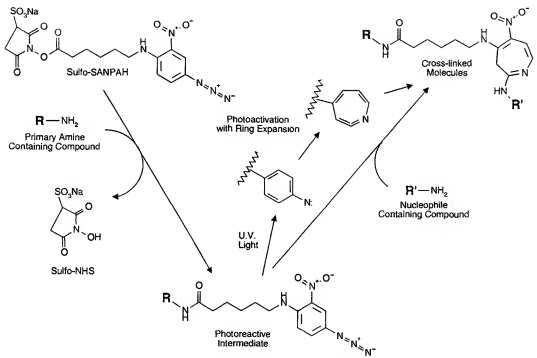


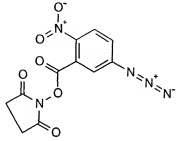
Figure 175 The reaction sequence of cross-linking with sulfo-SANPAH involves first derivatizing an amine-containing molecule using its NHS ester end to create an amide bond. Exposure to UV light then causes ring expansion to the dehydroazepine derivative, which can couple with amines to form the final conjugate.

affinity labeling of the *N*-formyl peptide receptor binding site of intact human polymorphonuclear leukocytes (Schmitt *et al.*, 1983), and the cross-linking of vasoactive intestinal peptide to receptors on intact human lymphoblasts (Wood and O'Dorisio, 1985).

A water-soluble version of this cross-linker also exists. Sulfosuccinimidyl-6-(4′-azido-2′-nitrophenylamino)hexanoate (sulfo-SANPAH) contains the negatively charged sulfonate group on its NHS ring, lending greater hydrophilicity to the compound.

3.5. ANB-NOS

N-5-Azido-2-nitrobenzoyloxysuccinimide (ANB-NOS) is a photoreactive, heterobifunctional cross-linker containing an amine-reactive NHS ester group (Pierce). Its cross-bridge is formed form a benzoic acid derivative, allowing molecules to be conjugated at relatively short 7.7-Å distances apart. The phenyl ring of ANB-NOS contains a nitro group that has the effect of shifting the optimal wavelength of activation to longer UV regions. The photoreaction is initiated by exposure to light in the range of 320–350 nm. Without the presence of the nitro group, activation would occur at much lower wavelengths, around 265–275 nm—wavelengths that potentially can damage biological molecules when exposed under high-photon irradiation. ANB-NOS typically is used to label an amine-containing protein or molecule by its NHS ester end. The resultant derivative is allowed to interact with other molecules that potentially can bind specifically to it and photolyzed to effect the final conjugation (Fig. 176).



ANB–NOS
N-5-Azido-2-nitrobenzoyloxy-
succinimide
MW 305.21
7.7 Å

Reported applications using this reagent include cross-linking of the aggregation state of cobra venom phospholipase A2 (Lewis *et al.*, 1977) and conjugation of the signal sequence of nascent preprolactin to a polypeptide of the signal recognition particle (Krieg *et al.*, 1986).

3.6. SAND

Sulfosuccinimidyl-2-(*m*-azido-o-nitrobenzamido)-ethyl-1,3′-dithiopropionate (SAND) is a heterobifunctional reagent containing an amine-reactive sulfo-NHS ester at one end and a photoreactive phenylazide group on the other (Pierce). The presence of the sulfonate group on the NHS ring lends water solubility to the reagent due to its

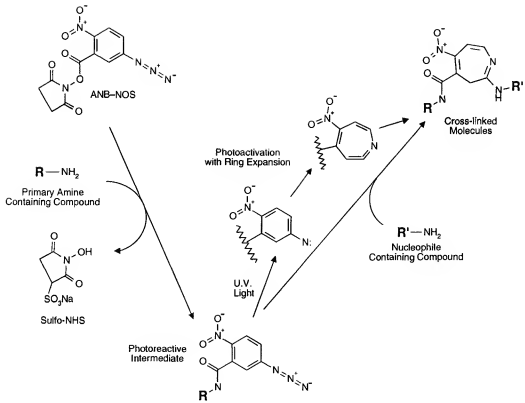
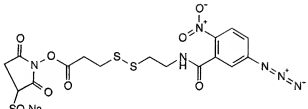


Figure 176 The NHS ester of ANB-NOS reacts with amines to form amide bonds. Subsequent photolyzing of the complex with UV light causes phenyl azide ring expansion and reaction with neighboring amines.

negative charge in aqueous solutions. In addition, the phenylazide group contains a nitro constituent that shifts the optimal range of photoactivation toward higher wavelengths—into the 320 to 350-nm region, thus decreasing the potential of photolytic damage to other sensitive groups that may be present during cross-linking. The extended cross-bridge of SAND (18.5 Å) provides a long spacer to accommodate even relatively distant sites between interacting molecules. The presence of a disulfide bond within the cross-bridge means that the reagent also is cleavable by the use of a disulfide reductant, allowing the potential for disruption of the cross-links after purification of the conjugate.

In use, SAND is first reacted with an amine-containing protein or other molecule—being careful to protect the photoreactive functional group from inadvertent degradation by exposure to excessive room light or sun. The modified intermediate then is allowed to interact with a target molecule. Finally, the photolyzing process is done to effect a nonselective cross-link between the modified molecule and any target molecules within van der Waals distance to the cross-linker (Fig. 177). Its use may be similar to that reported for sulfo-SANPAH, and its cleavability similar to that reported for SADP.



SAND
Sulfosuccinimidyl-2-(*m*-azido-*o*-nitro-benzamido)-ethyl-1,3'-dithiopropionate
MW 570.52
18.5 Å

3.7. SADP and Sulfo-SADP

N-Succinimidyl-4(4-azidophenyl)1,3'-dithiopropionate (SADP) is a photoreactive heterobifunctional cross-linker that is cleavable by treatment with a disulfide reducing agent (Pierce). The cross-linker contains an amine-reactive NHS ester and a photoactivatable phenylazide group, providing specific, directed coupling at one end and nonselective insertion at the other end.

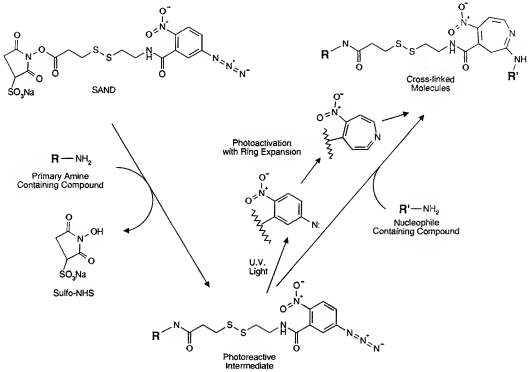
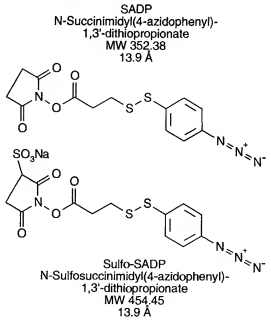


Figure 177 SAND can be used to modify amine-containing molecules, and then photoinitiate cross-linking to another amine-containing molecule via a ring-expansion process. The conjugates may be disrupted by reduction of the cross-bridge disulfide with DTT.

SADP is first used to modify a protein via its amine groups through the reactive NHS ester end of the cross-linker. After allowing for interaction of the modified protein with target molecules, the photoreactive group is used to couple with any molecules within van der Waals distance. The photolysis reaction requires UV exposure in the range of 265–275 nm to effect the final linkage. The presence of the disulfide group in SADP's cross-bridge allows disruption of cross-links with 50 m*M* DTT after the conjugation reaction is complete (Fig. 178).



SADP is hydrophobic and should be dissolved in organic solvent prior to addition of a small aliquot to an aqueous reaction. Concentrated stock solutions can be prepared in DMSO or DMF. Final concentration of the organic solvent in a cross-linking reaction should not exceed about 10% to prevent protein precipitation or denaturation.

Reported applications of SADP include the cross-linking of Con A to receptors on human erythrocyte membranes (Vanin and Ji, 1981), site-specific labeling of lectins using modified glycopeptides (Baenziger and Fiete, 1982), conjugation of a mouse cell-surface polypeptide with a Sendai virion envelope on newly infected cells (Zarling *et al.*, 1982), and cross-linking of platelet glycoprotein Ib (Jung and Moroi, 1983).

Sulfo-SADP is a water-soluble analog of SADP that contains a negatively charged sulfonate group on its NHS ring. The reagent may be added directly to aqueous reaction mixtures without prior dissolution in an organic solvent. Concentrated stock solutions prepared in water should be used immediately to prevent extensive hydrolysis of the sulfo-NHS ester group.

3.8. Sulfo-SAPB

Sulfosuccinimidyl 4-(*p*-azidophenyl)butyrate (sulfo-SAPB) is a photoreactive heterobifunctional reagent containing an amine-reactive sulfo-NHS ester at one end (Pierce).

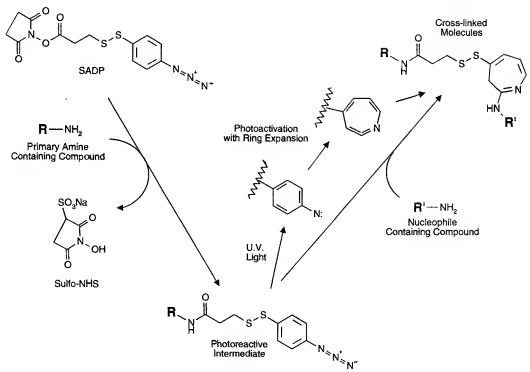
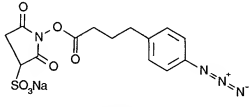


Figure 178 SADP reacts with amines via its NHS ester end to produce amide bonds. The modified molecule then may be photolyzed to create a nucleophile-reactive dehydroazepine intermediate able to covalently couple with amine-containing compounds.

The cross-linker is similar in design to sulfo-HSAB (Section 3.3), but containing a 3-carbon-longer cross-bridge. The sulfo-NHS ester provides water solubility to the reagent due to the negative charge of the sulfonate group. The phenylazide end can be photolyzed by exposure to UV light in the wavelength range of 265–275 nm (Fig. 179). Although there are no reported applications for the cross-linker, its reactivity and use is similar to that of sulfo-HSAB. The commercial availability of the reagent provides additional options for spacer length to study the interactions between two proteins or other molecules.



Sulfo-SAPB
Sulfosuccinimidyl-
4-(*p*-azidophenyl)butyrate
MW 404.32
12.8 Å

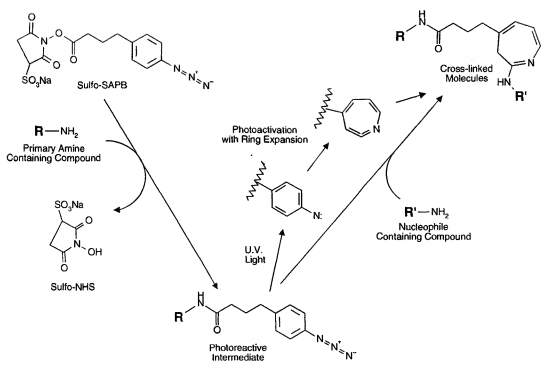
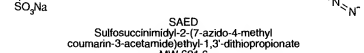


Figure 179 The reaction of sulfo-SAPB with an amine group is done first to form amide bond derivatives through its NHS ester end. Subsequent exposure to UV light causes the phenyl azide group to ring expand to a highly reactive dehydroazepine, which can couple to nucleophiles, such as amines.

3.9. SAED

Sulfosuccinimidyl 2-(7-azido-4-methylcoumarin-3-acetamide)ethyl-1,3'-dithiopropionate (SAED) is a photoreactive heterobifunctional cross-linking agent that also contains a fluorescent group (Pierce). The sulfo-NHS ester end of the reagent reacts with primary amines in proteins and other molecules to form stable amide linkages. The photoreactive end is an AMCA derivative (Chapter 8, Section 1.3) containing a light-sensitive azide group on the aromatic ring. Photolyzing with light in the range of long UV to within the visible spectrum will result in nonselective bond formation with nucleophiles and active carbon—hydrogen bonds within van der Waals distance (Fig. 180).



SAED
Sulfosuccinimidyl-2-(7-azido-4-methyl coumarin-3-acetamide)ethyl-1,3'-dithiopropionate
MW 621.6
23.6 Å

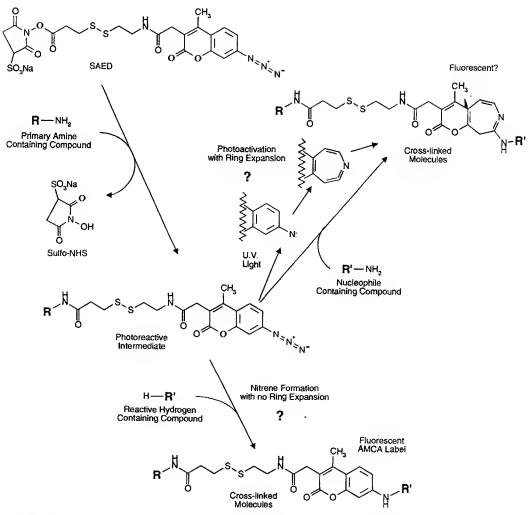


Figure 180 SAED can be used to modify amine-containing molecules through its NHS ester end. Subsequent exposure to UV light causes bond formation with nearby nucleophilic groups, such as amines. The photosensitive phenyl azide group is created on the aromatic ring of an AMCA fluorophore. Before photolysis, the azide group makes the cross-linker nonfluorescent. After photolyzing, however, the azide group is either lost by N_2 generation or couples to a target molecule. Either way, the AMCA portion becomes fluorescent to allow tracking of the conjugate. The photoreaction may occur through ring expansion to an intermediate dehydroazepine or might happen through nitrene formation.

SAED is a relatively large cross-linker containing a long (22.5 Å) cross-bridge. The central portion of its cross-bridge contains a disulfide bond, making the reagent susceptible to cleavage with disulfide reducing agents. The aromatic character of the coumarin derivative creates a maximal UV absorptivity at 327 nm with an extinction coefficient of 18,200 $M^{-1}cm^{-1}$ for a 1 mg/ml solution in acetonitrile:water (15:2 v/v). The extinction coefficient at 298 nm for the same concentration of SAED in the identical solvent is 13,625 $M^{-1}cm^{-1}$.

SAED contains a sulfo-NHS ester with a negatively charged sulfonate group on its ring. The presence of this negative charge does lend some expected water solubility to

the reagent (3 mg/ml at room temperature), but because of the reagent's large size it does not provide the same water solubility benefits as with other smaller cross-linkers. It is also sparingly soluble in acetonitrile (2.5 mg/ml), but only if a small amount of water is present (15:2 acetonitrile:water, v/v). However, SAED is very soluble in DMSO and DMF (about 50 mg/ml). Stock solutions may be prepared in dry DMSO or DMF while maintaining fairly good stability of the reagent's functional groups. The addition of a small quantity of these stock solutions to an aqueous reaction medium facilitates the amine-modification process via the sulfo-NHS ester end of the cross-linker. The final concentration of organic solvent in the aqueous reaction should not exceed 10%.

The coumarin derivative of SAED is not fluorescent until the photolysis reaction is initiated. A protein modified with SAED will fluoresce after activation with UV light whether or not the photoreactive end actually couples to the intended target, since breakdown of the azide group on the ring is all that is required to initiate fluorescence. Thus, the level of SAED incorporation into a macromolecule may be assessed by the resultant coumarin fluorescence after separation of the derivative from excess reagent. Native AMCA has an excitation optimum at 345–350 nm and an emission wavelength range of 440–460 nm. The excitation and emission properties of SAED may change somewhat upon its attachment to macromolecules due to fluorescent quenching; however, the coumarin tag will still remain fluorescently active even after cross-linking.

Since the cross-linker is cleavable, SAED provides a means of fluorescent transfer of the coumarin tag to a second molecule, which interacts with the initially modified protein (Fig. 181). For example, soybean trypsin inhibitor (STI) was labeled with SAED and then allowed to interact with trypsin. After photoreactive cross-linking of the two interacting molecules, the complex was reduced with DTT, breaking the conjugate and transferring the fluorescent tag to trypsin near the STI binding site (Thevenin *et al.*, 1991).

In another study, SAED was used to investigate the role of the foot protein moiety of the triad and its relationship to Ca^{2+} release from sarcoplasmic reticulum (Kang *et al.*, 1991). Modification of poly-L-lysine (a Ca^{2+} release inducer) and neomycin with the cross-linker was done followed by subsequent incubation with the foot protein and photoreactive conjugation. Cleavage of the cross-links with a disulfide reductant allowed transfer of the fluorescent tag to the foot protein in areas near the binding sites. Fluorescent monitoring of conformational changes within the protein upon varying the Ca^{2+} concentration was then possible.

Since the photoreactive cross-linking step with SAED occurs rapidly on exposure to even bright light within the visible spectrum, UV lamps are not required. However, special care should be taken to protect the reagent from exposure to light before the photolysis reaction is initiated. The solid should be stored in amber bottles and any stock solutions prepared in organic solvent should be wrapped to exclude light totally. In addition, the initial derivatization of an amine-containing molecule should be done in the dark in wrapped containers.

3.10. Sulfo-SAMCA

Sulfosuccinimidyl 7-azido-4-methylcoumain-3-acetate (sulfo-SAMCA) is a heterobifunctional reagent similar in design to SAED (this chapter, Section 3.9) (Pierce). One

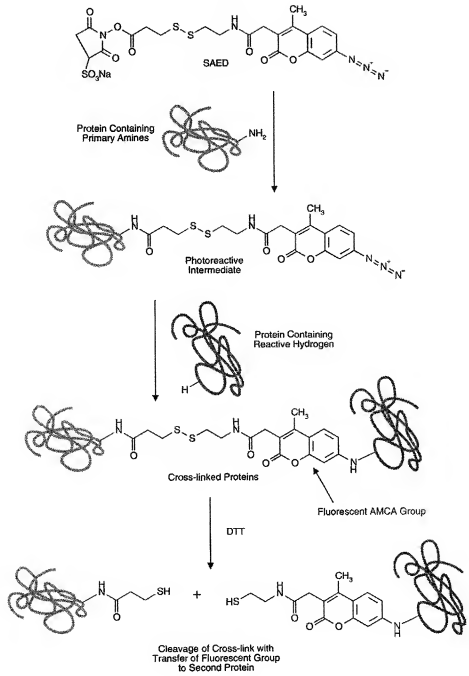
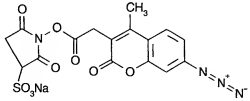


Figure 181 SAED may be used to transfer the fluorescent AMCA label from the first molecule modified with the cross-linker to the second molecule cross-linked with it by reduction of its internal disulfide bond with DTT. Thus, unknown target molecules may be fluorescently tagged to follow them *in vivo*.

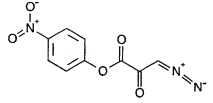
end of the cross-linker contains an amine-reactive sulfo-NHS ester, while the other end is an AMCA derivative (Chapter 2, Section 1.3) that contains a photosensitive phenylazide group. Unlike SAED, however, sulfo-SAMCA contains a short noncleavable cross-bridge (12.8 Å) where the active ester functional group is constructed directly off the carboxylate of AMCA acid without any other intervening spacer groups. Conjugated molecules will retain the fluorescent label, thus providing detectability to the complexes formed (Fig. 182). However, since cross-links formed with this reagent are not cleavable, sulfo-SAMCA cannot function as a fluorescent transfer agent in the fashion of SAED.



Sulfo-SAMCA
Sulfosuccinimidyl-7-azido-
4-methylcoumarin-3-acetate
MW 458.34
12.8 Å

3.11. *p*-Nitrophenyl Diazopyruvate

Diazopyruvates are a relatively new class of photoreactive reagents that can be used in heterobifunctional cross-linking experiments. The *p*-nitrophenyl ester derivative of diazopyruvate provides amine-reactive, acylating potential, while the photosensitive group can be activated with UV light to generate reactive aldehydes. More specifically, the diazo functional group can be photolyzed by exposure to irradiation at 300 nm, forming a highly reactive carbene that can undergo a Wolff rearrangement that produces a ketene amide intermediate. In the presence of a nucleophilic species on a target molecule, the ketene can undergo an acylation reaction to form a stable malonic acid derivative. The photolyzed product thus can couple to hydrazide- or amine-containing targets to form covalent linkages (Fig. 183).



pNPDP
p-Nitrophenyl diazopyruvate
MW 235

p-Nitrophenyl diazopyruvate (Molecular Probes) is relatively insoluble in water or aqueous buffers, but may be predissolved in DMF before adding an aliquot of the stock solution to an aqueous reaction mixture. All solutions of the reagent should be care-

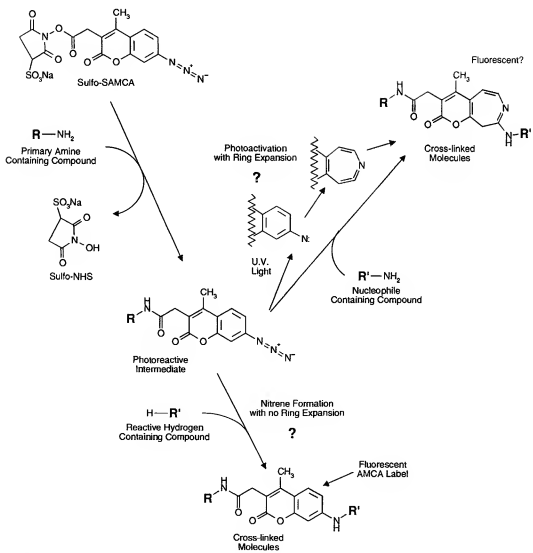


Figure 182 Sulfo-SAMCA can be used to modify amine-containing molecules through its NHS ester end. Subsequent exposure to UV light causes bond formation with nearby nucleophilic groups, such as amines. The photosensitive phenyl azide group is created on the aromatic ring of an AMCA fluorophore. Before photolysis, the azide group makes the cross-linker nonfluorescent. After photolyzing, however, the azide group is either lost by N_2 generation or couples to a target molecule. Either way, the AMCA portion becomes fluorescent to allow tracking of the conjugate. The photoreaction may occur through ring expansion to an intermediate dehydroazepine or might happen through nitrene formation.

fully protected from light to prevent premature photolysis. *p*-Nitrophenyl diazopyruvate has an absorbance maximum at 390 nm with a molar extinction coefficient of about 19,000 $M^{-1}cm^{-1}$ in methanol.

p-Nitrophenyl diazopyruvate has been used in the photoreactive cross-linking of

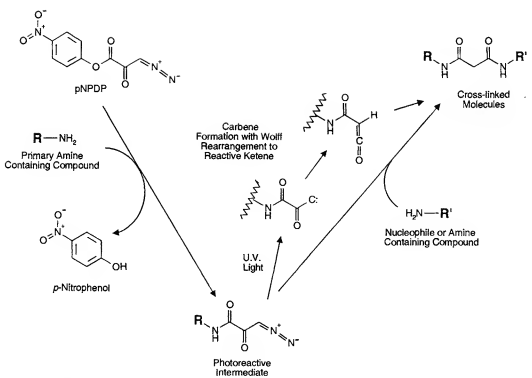


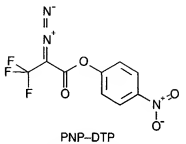
Figure 183 pNPDP reacts with amine-containing compounds by its *p*-nitrophenyl ester group to form amide bonds. After photolyzing the diazo derivative with UV light, a Wolff rearrangement occurs to a highly reactive ketene intermediate. This group can couple to nucleophiles such as amines.

calmodulin with adenylate cyclase from bovine brain (Harrison *et al.*, 1989) and to cross-link aldolase (Goodfellow *et al.*, 1989).

3.12. PNP-DTP

p-Nitrophenyl-2-diazo-3,3,3-trifluoropropionate (PNP-DTP) is a unique photoreactive heterobifunctional cross-linker that contains an amine-reactive group on one end and a photosensitive diazo group on the other (Chowdhry *et al.*, 1976) (Pierce) (Fig. 184). *p*-Nitrophenyl esters react in a manner similar to that of NHS esters (Chapter 4, Section 1, and Chapter 2, Section 1.4) with *p*-nitrophenol as the leaving group upon reaction with a nucleophile. Amine-containing target molecules such as proteins can be modified with this reagent to form amide bond derivatives possessing photoactivatable functional groups. The reagent is small enough to probe deep within the active centers of receptor molecules and other sites of biomolecular interactions.

PNP-DTP has been used to photoaffinity-label the thyroid hormone nuclear receptors in intact cells by preparing a derivative of 3,5,3′-triiodo-L-thyronine with the cross linker (Pascual *et al.*, 1982; Casanova *et al.*, 1984). Effective photoreactive conjugation was found to occur after irradiation with UV light at 254 or 310 nm.



PNP–DTP
p-Nitrophenyl-2-diazo-
3,3,3-trifluoropropionate
MW 276.15

4. Sulfhydryl-Reactive and Photoreactive Cross-linkers

The benefits of nonselective photoreactive cross-linking can be merged with the directed coupling ability of sulfhydryl-reactive functional groups to create heterobifunctional reagents possessing greater utility than the standard amine- and photoreactive agents discussed previously. Having a sulfhydryl-reactive group on one end of the

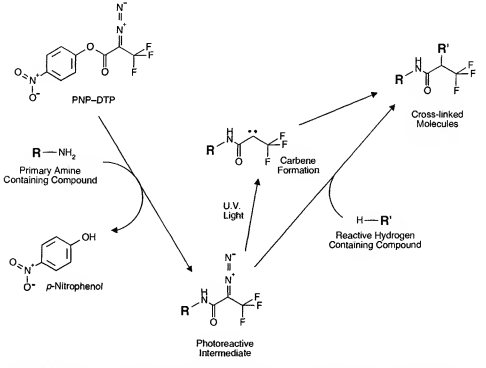


Figure 184 PNP-DTP can modify amine-containing molecules through its *p*-nitrophenyl ester group to form amide bonds. Exposure of its photosensitive diazo group with UV light generates a highly reactive carbene that can insert into active C—H or N—H bonds.

cross-linker allows the initial conjugation to take place at more limited sites on proteins and other molecules before irradiation to effect the final photosensitive reaction.

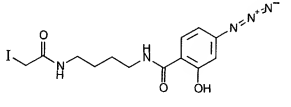
The following reagents contain a variety of sulfhydryl-reactive groups, including iodoacetyl derivatives, maleimide compounds, and pyridyl disulfide chemistries. The iodoacetyl and maleimide functions form permanent thioether bonds with target molecules containing free sulfhydryls. The pyridyl disulfide derivative reacts with —SH groups to form reversible disulfide linkages, which can be cleaved with disulfide reducing agents like DTT.

The photoreactive end of the following cross-linkers also varies from the traditional aryl azide group to the newer benzophenone and fluorinated aryl azide derivatives. The fluorinated phenyl azide functional groups photolyze to true nitrenes without the ring expansion side-reaction characteristic of aryl azides. The result is that fluorinated aryl azides more effectively insert into active carbon—hydrogen bonds, rather than potentially undergoing nucleophilic reactions like phenyl azides. Benzophenone groups generally have higher degrees of bond formation with the intended target molecule compared to the yields obtained using traditional phenyl azides, due to their ability to be repeatedly photolyzed without breakdown of the precursor to an inactive form.

The number of commercially available cross-linkers for sulfhydryl- and photoreactive conjugations provide enough variety to design successful experiments in photolabeling active centers and studying macromolecular interactions.

4.1. ASIB

1-(*p*-Azidosalicylamido)-4-(iodoacetamido)butane (ASIB) is a heterobifunctional cross-linker containing a sulfhydryl-reactive iodoacetyl group on one end and a photosensitive phenyl azide group on the other (Pierce). The phenyl azide ring is substituted with a ring-activating hydroxyl group that provides the ability to radioiodinate the compound before the conjugation reaction is performed. Since both the iodoacetyl and the phenyl azide functional groups are relatively stable in aqueous solutions, the steps involved in iodination and cross-linking do not detrimentally affect the subsequent reactivity of the reagent. All operations should be done protected from light, however, to prevent premature photolysis before the desired cross-linking reaction is initiated. The cross-bridge of the reagent provides a long 18.8-Å spacer between cross-linked molecules.



ASIB
1-(*p*-Azidosalicylamido)-
4-(iodoacetamido)butane
MW 417.21
18.8 Å

The reaction of ASIB with sulfhydryl-containing molecules can be done at mildly alkaline pH with excellent specificity. Higher pH conditions may cause cross-reactivity with amines. Photolyzing with UV light may result in immediate reaction of the nitrene intermediate with a target molecule within Van der Waals distance, or may result in ring expansion to the nucleophile-reactive dehydroazepine. The ring-expanded product is primarily reactive toward amine groups (Fig. 185).

4.2. APDP

N-[4-(*p*-Azidosalicylamido)butyl]-3′-(2′-pyridyldithio) propionamide (APDP) is a radioiodinatable, heterobifunctional cross-linking agent that contains a sulfhydryl-reactive pyridyl disulfide group on one end and a photosensitive phenyl azide on the other end (Pierce). Radioiodinatable cross-linkers eliminate the need to radiolabel one of the reacting proteins, thus avoiding potential activity losses due to modification of important residues (Chapter 2, Section 4.5). They also allow radiolabeling of unknown target molecules that interact with the initially modified protein. APDP reacts with sulfhydryl-containing proteins and other molecules to form a reversible disulfide bond. If the cross-linker is radiolabeled prior to conjugation, cleavage of the disulfide group with DTT after cross-linking effectively transfers the iodinated portion to the

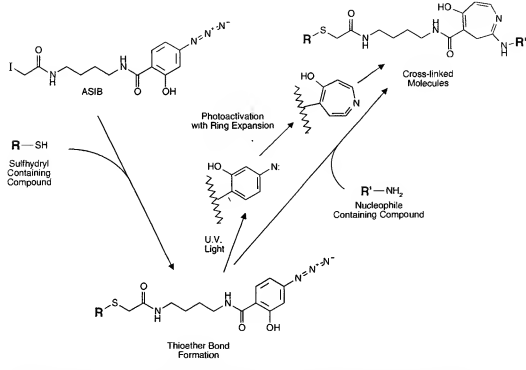
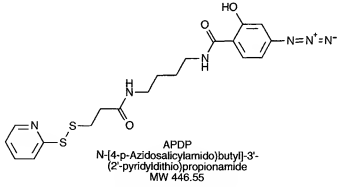


Figure 185 ASIB can react with sulfhydryl-containing molecules through its iodoacetate group to form thioether linkages. Subsequent exposure to UV light causes a ring-expansion process to occur, creating a highly reactive dehydroazepine intermediate that can couple to amine-containing molecules.

secondary, photocoupled protein. This radiolabel transfer process allows tracking of a specific receptor or other interacting species after conjugation with its complementary ligand (Fig. 186).



The reactions of APDP are similar to that of the reported compound *N*-(4-azidophenyl)thiophthalimide, a nonradioiodinatable cross-linker (Moreland *et al.*, 1982). Both the phenyl azide group and the pyridyl disulfide portion are stable in

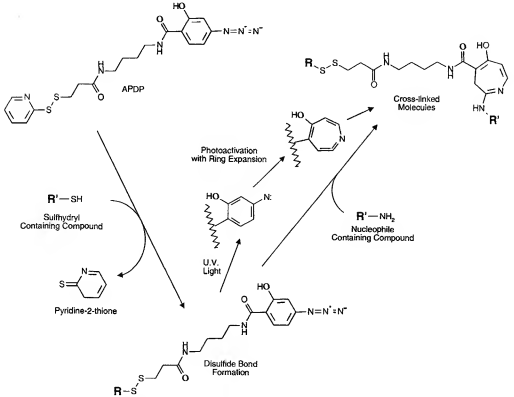


Figure 186 APDP can modify sulfhydryl-containing compounds through its pyridyl disulfide group to form disulfide bonds. Its phenyl azide end then can be photolyzed with UV light to couple with nucleophiles via a ring expansion process. The disulfide group of the cross-link can be selectively cleaved using DTT.

aqueous environments prior to the cross-linking reaction. The initial modification with a sulfhydryl-containing protein should be done protected from light to preserve the activity of the photosensitive functional group. Avoid, also, in the reaction medium disulfide reducing agents that can react with the pyridyl disulfide group as well as inactivate the phenyl azide portion.

The cross-bridge of APDP provides an extremely long, 21.02-Å spacer that is able to reach distant points between two interacting molecules. Cleavage of the cross-link with a disulfide reducing agent regenerates the original sulfhydryl-modified protein without leaving any other chemical groups behind. The remainder of the cross-linker stays attached to the second, photolabeled protein.

APDP is soluble in DMSO and DMF, but almost insoluble in acetone or water. Stock solutions may be prepared in DMSO or DMF and a small aliquot added to an aqueous reaction mixture. Do not exceed 10% organic solvent in the buffered reaction. Both functional groups of APDP will react in a variety of salt conditions and pH values. For reaction with a sulfhydryl-containing protein, a buffer at physiological pH containing a metal chelating agent to protect the free sulfhydryl groups from oxidation is recommended (i.e., 0.01–0.1 *M* sodium phosphate, 0.15 *M* NaCl, pH 7.2, containing 10 m*M* EDTA).

Iodination of the cross-linker may be done according to the procedures discussed in Chapter 8, Section 4, or similar to that described for SASD (this chapter, Section 3.2).

4.3. Benzophenone-4-iodoacetamide

A photoreactive group consisting of a benzophenone residue photolyzes upon exposure to UV light to give a highly reactive triplet-state ketone intermediate (Walling and Gibian, 1965). Similar to the reactive nitrene of photolyzed phenyl azides, the energized electron of an activated benzophenone can insert in active hydrogen—carbon bonds and other reactive groups to give covalent linkages with target molecules. Unlike phenyl azides, however, the decomposition or decay of the photoactivated species does not yield an inactive compound. Instead, benzophenones that have become deactivated without forming a covalent bond can be once again photolyzed to an active state. The results of this multiple-activation characteristic are more than one chance to form a cross-link with the intended target and much higher yields of photocross-linking.

The heterobifunctional cross-linker benzophenone-4-iodoacetamide is a photoreactive reagent containing a sulfhydryl-reactive iodoacetyl derivative at one end and a benzophenone group on the other end (Hall and Yalpani, 1980; Tao *et al.*, 1984; Lu and Wong, 1989) (Molecular Probes). The iodoacetyl group has reactivity similar to that of the same group on the heterobifunctional reagent SIAB (this chapter, Section 1.5). Under alkaline pH conditions (pH 8–9) the iodoacetyl reaction is highly specific for sulfhydryl residues in proteins and other molecules, forming stable thioether linkages. The initial modification reaction of sulfhydryl-containing compounds should be done protected from light to avoid premature photolysis of the benzophenone functionality. After purification of the modified protein from excess reagent (by dialysis or gel filtration) mix the modified protein with a second target molecule, allow the interaction to take place, and photolyze with UV light to effect the final cross-link (Fig. 187). Since repeated photolysis of the benzophenone species is possible, the yield of

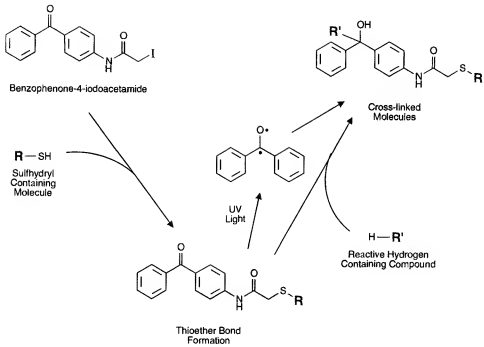
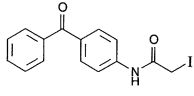


Figure 187 Benzophenone-4-iodoacetamide reacts with sulfhydryl-containing compounds to give thioether linkages. Subsequent photolyzing of the benzophenone residue gives a highly reactive triplet-state ketone intermediate. The energized electron can insert in active C—H or N—H bonds to give covalent cross-links.

such conjugation reactions can be significantly higher than that when using other photoreactive groups. One report indicated that cross-linking with chymotrypsin approached 100% efficiency (Campbell and Gioannini, 1979).



Benzophenone-4-iodoacetamide
MW 365

Benzophenone-4-iodoacetamide is water-insoluble and should be predissolved in DMF or another organic solvent prior to adding an aliquot to an aqueous reaction mixture. Stock solutions may be prepared and stored successfully if protected from light.

4.4. Benzophenone-4-maleimide

Benzophenone-4-maleimide is a heterobifunctional photoreactive cross-linker that has sulfhydryl reactivity similar to benzophenone-4-iodoacetamide discussed in the

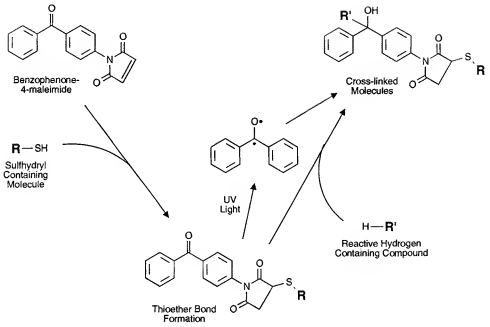
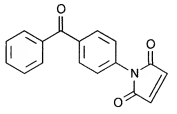


Figure 188 Benzophenone-4-maleimide can couple to thiol-containing molecules to form stable thioether bonds. Exposure of the benzophenone group to UV light causes transition to a triplet-state ketone of high reactivity for insertion into C—H or N—H bonds.

previous section (Molecular Probes). In this case, the sulfhydryl-reactive portion is provided by the presence of a maleimide functional group, which couples to —SH groups by addition to the double bond (Chapter 2, Section 2.2). The maleimide group is reasonably specific for sulfhydryls, and the reaction results in a thioether linkage that is quite stable. Sulfhydryl-containing proteins and other molecules modified with this reagent may be used in photoaffinity-labeling studies to investigate the specific interactions between two molecules. After mixing, the solution may be photolyzed to create a covalent cross-link between the two interacting substances. Ultraviolet photolysis of the benzophenone group results in a highly reactive triplet-state intermediate that can rapidly insert or add to organic functional groups within van der Waals distance (Fig. 188). Decay of the active-state intermediate returns the photosensitive group to its orignial chemical form, thus allowing repeated photoactivations without losing the potential for coupling to its intended target.



Benzophenone-4-maleimide
MW 277

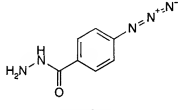
Benzophenone-4-maleimide is water-insoluble and should be predissolved in DMF or another organic solvent prior to adding an aliquot to an aqueous reaction mixture. Stock solutions may be prepared and stored successfully if protected from light. The hydrophobicity and bulkiness of the benzophenone group may cause insolubility problems in the initial protein that is modified if the derivatization is done at too high a level. Fortunately, the use of a sulfhydryl-reactive reagent can limit the degree of derivatization, since —SH groups usually are present in lower quantities and in more discrete locations than groups like amines.

5. Carbonyl-Reactive and Photoreactive Cross-linkers

Cross-linking reagents containing a photoreactive function on one end and a carbonyl-reactive group on the other end are rare. The use of an amine group on one end of a photosensitive heterobifunctional reagent has been described (Gorman and Folk, 1980; Drafler and Marinetti, 1977; Das and Fox, 1979), but the presence of a hydrazide is required for spontaneous reactivity toward carbonyls. The following compound is the only commercially available reagent containing a phenyl azide photoreactive group and a hydrazide functional group.

5.1. ABH

p-Azidobenzoyl hydrazide (ABH) is a small, heterobifunctional cross-linker containing a photoreactive phenyl azide group on one end and a hydrazide functional group on the other end (Pierce). The hydrazide can react with carbohydrate-containing molecules after oxidation with sodium periodate (Chapter 1, Section 4.4) to create aldehyde residues. The reaction forms a hydrazone linkage. Thus, glycoproteins may be specifically labeled on their polysaccharide chains for subsequent investigation of their interaction with receptor molecules (Fig. 189). In this sense, lectin–carbohydrate interactions may be studied through direct modification of the sugar groups at or adjacent to the binding site. Other amine- or sulfhydryl-reactive probes may not be suitable for such studies due to the lack of amine or sulfhydryl groups near enough to a polysaccharide structure.



ABH
p-Azidobenzoyl hydrazide
MW 177.17
11.9 Å

The cross-bridge of ABH consists of a benzoic acid derivative and thus provides a short spacer between conjugated molecules. After ABH modification of a glycoprotein and incubation with a potential target molecule, the solution may be photolyzed with

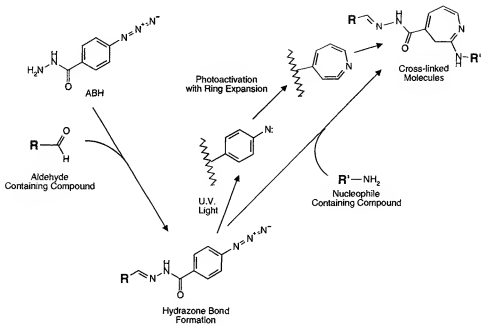


Figure 189 ABH reacts with aldehyde-containing compounds through its hydrazide end to form hydrazone linkages. Glycoconjugates may be labeled by this reaction after oxidation with sodium periodate to form aldehyde groups. Subsequent photolysis with UV light causes photoactivation of the phenyl azide to a nitrene. The nitrene undergoes rapid ring expansion to a dehydroazepine, which can couple to nucleophiles, such as amines.

UV light to initiate the final cross-link. Prior to photolysis, the reagent and all modified species should be protected from light to prevent degradation of the phenyl azide group.

ABH is relatively insoluble when directly added to water or buffer, and therefore it should be predissolved in DMSO prior to addition of an aliquot to an aqueous reaction medium. Stock solutions at a concentration of 50 m*M* ABH in DMSO work well. Since both functional groups of ABH are stable in aqueous environments as long as the solution is protected from light, a secondary stock solution may be made from the initial organic preparation by adding an aliquot to the hydrazide reaction buffer (0.1 *M* sodium acetate, pH 5.5; O'Shannessy and Quarles, 1985; O'Shannessy *et al.*, 1984). Make a 1:10 dilution of the ABH/DMSO solution in the reaction buffer. This solution may be stored in the dark at 4°C without decomposition.

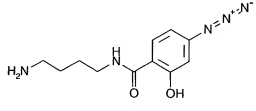
6. Carboxylate-Reactive and Photoreactive Cross-linkers

A carboxylate-reactive cross-linking compound typically contains a primary amine functional group that can be coupled to a carboxylic acid group in a protein or other molecule through the use of a suitable activating agent, such as a carbodiimide. The carbodiimide forms an active ester intermediate that then reacts with the amine to create an amide bond (Chapter 3, Section 1). Recent reported use of diazoalkyl deriva-

tives that spontaneously react with carboxylates have been tried with fluorescent probes, but not yet applied to heterobifunctional cross-linking agents (DeMar *et al.*, 1992; Schneede and Ueland, 1992) (Chapter 2, Section 3.1). The following heterobifunctional reagent is the only carboxylate-reactive photosensitive cross-linker currently available commercially.

6.1. ASBA

4-(*p*-Azidosalicylamido)butylamine (ASBA) is a carboxylate-reactive cross-linking agent containing a primary amine on one end and a photosensitive phenyl azide group on the other (Pierce). The cross-linker is not spontaneously reactive with carboxylates, but must be used with another agent that facilitates bond formation. For instance, it can be used in conjunction with a carbodiimide or other such reagent system that can initiate covalent bond formation with a recipient carboxylic acid. A water-soluble carbodiimide like EDC (Chapter 3, Section 1.1) is able to activate the carboxylates on a target protein, forming active ester intermediates (Fig. 190). In the presence of ASBA, derivatization will occur resulting in amide bond formation, thus leading to modification with a photoreactive group.



ASBA
4-(*p*-Azidosalicylamido)
butylamine
MW 249.27
16.3 Å

The cross-bridge of ASBA provides a reasonably long spacer (16.3 Å). The phenyl azide portion is constructed from a salicylic acid derivative and thus possesses a ring-activating hydroxyl group. The presence of this group allows radioiodination of the ring prior to cross-linking (Chapter 8, Section 4.5).

Before the photolyzing step is initiated, the reagent should be handled in the dark or protected from light to avoid decomposition of the phenyl azide group.

7. Arginine-Reactive and Photoreactive Cross-linkers

The guanidinyl group on arginine's side chain can be specifically targeted by the use of 1,2-dicarbonyl reagents,such as the diketone group of glyoxal (Chapter 2, Section 5.2). Under alkaline conditions, this type of group can condense with the guanidinyl residue to form a Schiff base-like complex. The presence of other chemical compounds in the reaction can cause further structural rearrangements, such as stabilization by boronate (Pathy and Smith, 1975). Derivatives such as phenylglyoxal and *p*-nitro-

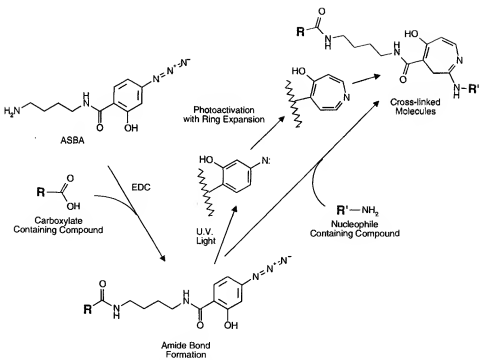


Figure 190 ASBA contains a primary amine group that may be conjugated to carboxylate compounds using the carbodiimide EDC. Subsequent exposure to UV light initiates the photoreaction leading to covalent cross-links.

phenylglyoxal can be used to block or quantitatively determine the amount of arginine in a protein (Yamasaki *et al.*, 1981). Studies have shown that if the reaction is done with a 2:1 ratio of glyoxal compound to arginine residues then the modification that results is reversible (Takahashi, 1968). If the modification is done at a 1:1 stoichiometry, then it is irreversible (Konishi and Fujioka, 1987).

The ability to direct conjugation or modification specifically through arginine residues using this chemistry has been exploited in the availability of the only photoreactive glyoxal derivative, APG.

7.1. APG

p-Azidophenyl glyoxal (APG) is a heterobifunctional cross-linker containing an arginine-specific diketone group on one end and a photosensitive phenyl azide group on the other end (Pierce). The reagent is a derivative of phenylglyoxal, a compound long used as an arginine guanidinyl modifier. Reaction of APG with proteins at pH 7–8 results in selective modification of arginine, leaving photoreactive groups available for subsequent cross-linking with interacting molecules (Fig. 191). Exposure to UV light effects the final cross-link. The cross-bridge of an APG cross-link is only 9.3 Å in

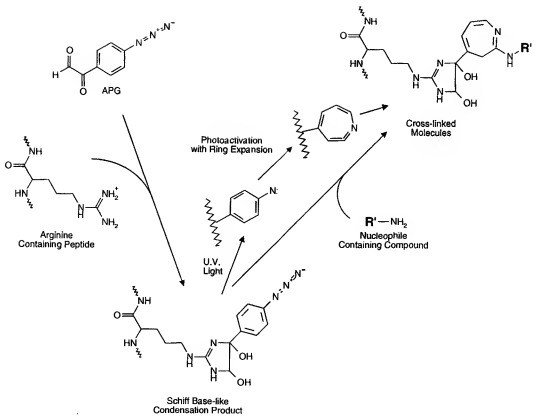
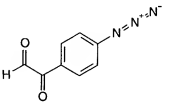


Figure 191 APG can be used to label specifically arginine residues in proteins, producing stable Schiff base interactions with the side-chain guanidino groups. Photolyzing with UV light then causes ring expansion of the phenyl azide group, initiating covalent bond formation with amines.

length, allowing proximity interactions to be studied or the irreversible labeling of arginine areas in proteins.



APG
p-Azidophenyl glyoxal
MW 193.16
(as the monohydrate)
9.3 Å

APG has been used to inhibit bovine heart lactic dehydrogenase, egg white lysozyme, horse liver alcohol dehydrogenase, and yeast alcohol dehydrogenase (Ngo *et al.*, 1981), in cross-linking RNA–protein interactions in *E. coli* ribosomes (Politz *et al.*, 1981), and in identifying regions of brome mosaic virus coat protein chemically cross-linked *in situ* to viral RNA (Sgro *et al.*, 1986).

ANNEX C

9

Preparation of Hapten–Carrier Immunogen Conjugates

This chapter describes the design, preparation, and use of hapten–carrier conjugates used to elicit an immune response toward a coupled hapten. The chemical reactions discussed for these conjugations are useful for coupling peptides, proteins, carbohydrates, oligonucleotides, and other small organic molecules to various carrier macromolecules. The resultant conjugates are important in antibody production, in immune response research, and in the creation of vaccines.

1. The Basis of Immunity

The essence of adaptive immunity is the ability of an organism to react to the presence of foreign substances and produce components (antibodies and cells) capable of specifically interacting with and protecting the host from their invasion. An "*antigen*" or "*immunogen*" is the name given for a substance that is able to elicit this type of immune response and also is capable of interacting with the sensitized cells and antibodies that are manufactured against it.

The immune system has two basic components that respond to a challenge of a foreign substance: a cellular response mediated by T lymphocytes and a humoral response mediated by secreted proteins called antibodies produced by B lymphocytes, also called plasma cells. The B lymphocytes recognize antigens through cell-surface immunoglobulins that bind to discrete chemical and structural epitopes on the antigen molecule. Each B cell possesses surface immunoglobulin of a single type (i.e., is monoclonal) and has a binding capacity that is directed against a discrete epitopic target.

Antigen binding by a complementary immunoglobulin molecule on the surface of B cells starts a process of cellular internalization of the foreign substance by pinocytosis. Once internalized by endosomes, systematic processing of the antigen takes place that breaks it down into smaller components.

At this point, the endosome may fuse with vesicles containing newly synthesized or recycling major histocompatibility complex (MHC) antigens. Some of the partially degraded antigenic fragments may form a complex with the MHC and be transported back to the cell surface. There they are "presented" to the circulating T-helper (T_h)

cells, which contain receptors able to bind specifically to particular structural and chemical characteristics of the degraded antigen–MHC complex. If a T_h cell recognizes and binds to the presented antigen on the surface of the APC, the T_h cell proliferates and begins to produce various lymphokines. Finally, the recognition and binding of the presented antigen by the T_h cells, coupled with the release of lymphokines, stimulate the associated B cells to proliferate and produce antibodies that recognize the intact antigen (Germain, 1986).

Antigens usually are macromolecules that contain distinct antigenic sites or "*epitopes*" that are recognized and interact with the various components of the immune system. They can exist as individual molecules composed of synthetic organic chemicals, proteins, lipoproteins, glycoproteins, RNA, DNA, or polysaccharides—or they may be parts of cellular structures (bacteria or fungi) or viruses (Harlow and Lane, 1988a,b,c; Male *et al.,* 1987).

Small molecules like short peptides, although normally able to interact with the products of an immune response, often cannot cause a response on their own. These "*haptens,*" as they are called, actually are incomplete antigens, and although not able by themselves to cause immunogenicity or to elicit antibody production, they can be made immunogenic by coupling them to a suitable carrier molecule (Fig. 270). Carriers typically are antigens of higher molecular weight that are able to cause an immunological response when administered *in vivo*.

In an immune response, antibodies are produced and secreted by the B lymphocytes in conjunction with the T_h cells. In the majority of hapten–carrier systems, the B cells end up producing antibodies that are specific for both the hapten and the carrier. In these cases, the T lymphocytes will have specific binding domains on the carrier, but will not recognize the hapten alone. In a kind of synergism, the B and T cells cooperate to induce a hapten-specific antibody response. After such an immune response has taken place, if the host is subsequently challenged with only the hapten, usually it will respond by producing hapten-specific antibodies from memory cells formed after the initial immunization.

Synthetic haptens mimicking some critical epitopic structures on larger macromolecules are often conjugated to carriers to create an immune response to the larger "parent" molecule. For instance, short peptide segments can be synthesized from the known sequence of a viral coat protein and coupled to a carrier to induce immu-

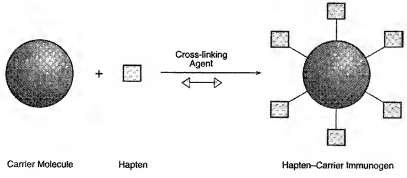


Figure 270 Immunogens are made by the cross-linking of a hapten molecule with a carrier using a conjugation reagent.

nogenicity toward the native virus. This type of synthetic approach to immunogen production has become the basis of much of the current research into the creation of vaccines.

The complete picture of the immune system is much more complex than this brief discussion can justly describe. In many instances, merely creating a B-cell response by using synthetic peptide–carrier conjugates, however well designed, will not always guarantee complete protective immunity toward an intact antigen. The immune response generated by a short peptide epitope from, say, a larger viral particle or bacterial cell may only be sufficient to generate memory at the B cell level. In these cases it is generally now accepted that a cytotoxic T-cell response is a more important indicator of protective immunity. Designing peptide immunogens with the proper epitopic binding sites for both B-cell and T-cell recognition is one of the most challenging research areas in immunology today.

Hapten–carrier conjugates also are being used to produce highly specific monoclonal antibodies that can recognize discrete chemical epitopes on the coupled hapten. The resulting monoclonals often are used to investigate the epitopic structure and interactions between native proteins. In many cases, the haptens used to generate these monoclonals are again small peptide segments representing crucial antigenic sites on the surface of larger proteins. Monoclonals developed from known peptide sequences will interact in highly defined ways with the protein from which the sequence originated. These antibodies then can be used, for example, as competitors to the natural interactions between a receptor and its ligand. Thus, using antibodies generated from hapten–carrier conjugates, information can be obtained about the precise sites of binding between macromolecules.

2. Types of Immunogen Carriers

The most commonly used carriers are all highly immunogenic, large molecules that are capable of imparting immunogenicity to covalently coupled haptens. Some of the more useful ones are proteins, but other carriers may be composed of lipid bilayers (liposomes), synthetic or natural polymers (dextran, agarose, poly-L-lysine), or synthetically designed organic molecules. The criteria for a successful carrier molecule are the potential for immunogenicity, the presence of suitable functional groups for conjugation with a hapten, reasonable solubility properties even after derivatization—although this is not an absolute requirement, since precipitated molecules can be highly immunogenic—and lack of toxicity *in vivo*.

Some synthetic carriers actually are designed to have low immunogenicity on their own to minimize the potential for antibody production against themselves. When a hapten is coupled to these molecules, the immune response is directed principally toward the modification, not at the carrier. This design approach guides most of the immune response toward the desired target and minimizes the production of carrier-specific antibodies.

2.1. Protein Carriers

The first carrier molecules used for immunogen conjugation were proteins. A foreign protein administered *in vivo* by any one of a number of potential routes nearly ensured

the elicitation of an immune response. In addition, protein carriers could be chosen to be highly soluble and possessed of abundant functional groups that could facilitate easy conjugation with a hapten molecule. When proteins are used as carriers in immunogen formation, the conjugates can be injected in any animal except the animal of origin for the carrier protein itself. In other words, the use of BSA would not be suitable for administration into cows, since self-proteins would not be expected to elicit good immune responses, even when attached with hapten molecules.

The most common carrier proteins in use today are keyhole limpet hemocyanin (KLH; MW 4.5×10^5 to 1.3×10^7), bovine serum albumin (BSA; MW 67,000), aminoethylated (or cationized) BSA (cBSA), thyroglobulin (MW 660,000), ovalbumin (OVA; MW 43,000), and various toxoid proteins, including tetanus toxoid and diphtheria toxoid. Other proteins occasionally used include myoglobin, rabbit serum albumin, immunoglobulin molecules (particularly IgG) from bovine or mouse sera, tuberculin-purified protein derivative, and synthetic polypeptides such as poly-L-lysine and poly-L-glutamic acid.

KLH

Perhaps the most popular carrier protein is KLH. The hemocyanin from keyhole limpets (the mollusk *Megathura crenulata*) is the oxygen-carrying protein of these primitive sea creatures. KLH is an extremely large, multisubunit protein that contains chelated copper of non-heme origin. In concentrated solutions above pH 7, it displays a characteristic opalescent blue color that betrays its near insolubility and copper prosthetic groups. In acidic solutions the blue color changes to green. At physiological pH the protein exists in various subunit aggregate states of large molecular weight. For instance, in Tris buffer at pH 7.4 it is known to associate in five different aggregate forms (Senozan *et al.*, 1981). In highly alkaline or acidic environments, KLH disassociates into subunits (Hersckovits, 1988). The protein exhibits increased immunogenicity when it is disassociated into subunits, probably due to exposure of additional epitopic sites to the immune system (Bartel and Campbell, 1959). The intact protein usually creates considerable light-scattering or iridescent effects due to its size and almost colloidal nature in aqueous solutions.

Since keyhole limpets are marine creatures existing in a high-salt environment, KLH maintains its best stability and solubility in buffers containing at least 0.9 *M* NaCl (not 0.9%). As the concentration of NaCl is decreased below about 0.6 *M*, the protein begins to precipitate and denature. Conjugation reactions using KLH, therefore, should be done under high-salt conditions to preserve the solubility of the hapten–carrier complex.

KLH also should not be frozen. Freeze–thaw effects cause extensive denaturation and result in considerable amounts of insolubles. Commercial preparations of KLH are typically freeze-dried solids that no longer fully dissolve in aqueous buffers and do not display the protein's typical blue color due to loss of chelated copper. The partial denatured state of these products often makes conjugation reactions difficult. Pierce Chemical is the only commercial source of KLH that includes special (proprietary) stabilizers to provide the protein in a lyophilized form that is almost completely soluble upon reconstitution and with its blue copper-binding characteristics still intact. Reconstitution of the Pierce product with water yields a buffered solution ready for conjugation reactions.

KLH contains an abundance of functional groups available for conjugation with hapten molecules. On a per-mole basis (using an average MW of 5,000,000), KLH has over 2000 amines from lysine residues, over 700 sulfhydryls from cysteine groups, and over 1900 tyrosines. Activation of the protein with SMCC (Section 5) typically results in 300–600 maleimide groups per molecule for coupling to sulfhydryl-containing haptens.

The preparation of immunogen conjugates often requires the coupling of a sparingly soluble hapten to a carrier molecule. Predissolving the hapten in an organic solvent and adding an aliquot of this solution to an aqueous reaction mixture typically is done to maintain at least some solubility of the hapten in the conjugation solution. DMSO may be used for this purpose with KLH while maintaining very good solubility characteristics of the protein as well as the hapten. KLH is completely soluble in 50% (v/v) DMSO, becomes cloudy at a level of 60%, and definitely precipitates at 67%. Therefore, conjugation reactions may be done by adding a volume of aqueous KLH to an equal volume of hapten dissolved in DMSO. Care should be taken, however, to avoid buffer salt precipitation on addition of organic solvent.

BSA and cBSA

BSA (MW 67,000) and cationized BSA (cBSA) are highly soluble proteins containing numerous functional groups suitable for conjugation. Even after extensive modification with hapten molecules these carriers usually retain their solubilities. The exception to this statement is when hydrophobic peptides or other sparingly soluble molecules are conjugated to the proteins. Modification of any carrier with hydrophobic haptens may cause enough masking of the hydrophilic surface to result in precipitation. Depending on the degree of precipitation, such conjugates often are useful in generating an immune response. To limit the production of insoluble complexes, however, the conjugation reaction can be sealed back to reduce the level of carrier modification.

BSA possesses a total of 59 lysine ε-amine groups (with only 30–35 of these typically available for derivatization), 1 free cysteine sulfhydryl (with 17 disulfides buried within its three-dimensional structure), 19 tyrosine phenolate residues, and 17 histidine imidazolides. The presence of numerous carboxylate groups gives BSA its net negative charge (pI 5.1).

Cationized BSA is prepared by modification of its carboxylate groups with ethylene diamine (Chapter 1, Section 4.3) (Fig. 271). Controlled aminoethylation using the water-soluble carbodiimide EDC results in blocking many of BSA's aspartic and glutamic acid side chains (and possibly the C-terminal carboxylate), forming an amide bond with an alkyl spacer containing a terminal primary amine group. Since the negative charge contributions of the native carboxylates are masked and positively charged amines are created in their place, the result of this process is a significant rise in the protein's pI. Cationization performed according to published procedures alters the net charge of BSA from a pI of about 5.1 (Cohn *et al.*, 1947) to over pI 11 (Muckerheide *et al.*, 1987a).

The highly positive charge of cBSA dramatically increases its immunogenicity. The positive character of the molecule aids in its binding to antigen presenting cells (APC) *in vivo*, the first step in antibody production. The protein thus gets incorporated into the APCs faster than molecules having lower pH values. It also gets processed at an

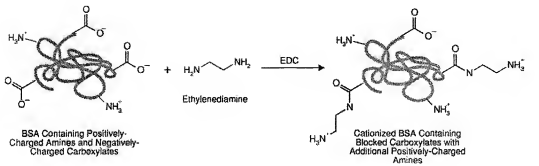


Figure 271 Cationized BSA is formed by the reaction of ethylene diamine with bovine serum albumin using the water-soluble carbodiimide EDC. Blocking of the carboxylate groups on the protein combined with the addition of terminal primary amines raises the pI of the molecule to highly basic values.

accelerated rate, producing a quicker immune response, and one that occurs with greater concentrations of specific antibody (Muckerheide *et al.*, 1987b; Domen *et al.*, 1987; Apple *et al.*, 1988; Domen and Hermanson, 1992).

Cationized BSA used as a carrier protein also induces a similar increase in the production of antibody against any attached hapten molecules. Even when haptens are coupled through cBSA's amine residues, the overall charge of the molecule remains basic enough to augment the immune response beyond that usually obtained using other carriers. This augmentation occurs even when the attached molecule is not merely a hapten, but a larger antigen macromolecule. Conjugation of a complete antigen (a molecule able to generate an immune response on its own) to cBSA causes an increased immune response against the antigen beyond that normally obtainable

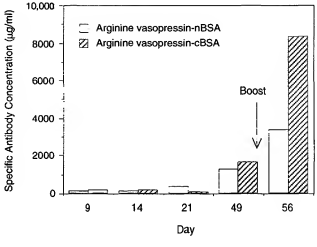


Figure 272 The effectiveness of cBSA as an immunogen can be seen by the comparison of specific antibody response in mice to arginine vasopressin coupled to both native and cationized BSA. The quantity injected was standardized according to the amount of arginine vasopressin present. The cationized carrier results in higher concentrations of antibody produced against the peptide than the immunogen made with native BSA.

for the native antigen administered in unconjugated form (Domen and Hermanson, 1992).

The effectiveness of cBSA as a carrier for peptides was investigated using arginine vasopressin (AV) as the hapten. Figure 272 shows the antibody concentration resulting after injection of the AV–cBSA conjugate intraperitoneally (ip) into BDF_1 female mice. As a control, native BSA was similarly conjugated with AV and administered in a second set of mice under identical conditions. The antibody concentration in the sera were monitored periodically by ELISA. The antibody response resulting from a set of mice injected with unconjugated peptide was subtracted in all cases. All injections were done using 100 μg of conjugate mixed with an equal volume of alum (22.5 mg/ml aluminum hydroxide) as adjuvant.

After the boost, the group of mice receiving the AV–cBSA conjugate generated over twice the antibody response as the group receiving the peptide conjugated to native BSA.

In a similar study, OVA conjugated to cBSA was compared to the same protein conjugated to native BSA (nBSA) and also OVA administered in an unconjugated form in mice. Figure 273 shows that before and after the boost, the OVA–cBSA conjugate resulted in much higher antibody concentrations than either the OVA–nBSA conjugate or OVA injected in an unconjugated form. Similar results were obtained for a conjugate of human IgG with cBSA (Fig. 274).

A corollary to the use of cBSA as a carrier protein is that its increased immune response often abrogates the use of complete Freund's adjuvant, now a source of concern because of its potential side effects in animals. A relatively innocuous mixture with alum is usually all that is required as adjuvant to result in good antibody production.

As mentioned previously for KLH, DMSO may be used to solubilize hapten molecules that are rather insoluble in aqueous environments. Conjugation reactions may be done in solvent/aqueous phase mixtures to maintain some solubility of the hapten

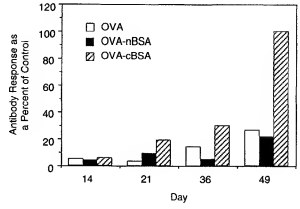


Figure 273 Cationized BSA even can increase the specific antibody response to large proteins coupled to it. This graph shows a comparison of the relative antibody response in mice to injections of ovalbumin, either in an unconjugated form or conjugated to native or cationized BSA. The quantity injected was standardized according to the amount of ovalbumin present. The highly basic cBSA molecule modulates the immune response to enhance the production of antibodies toward even proteins conjugated with it.

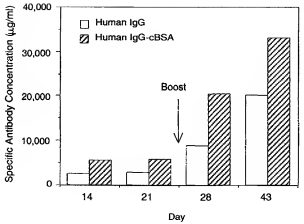


Figure 274 Human IgG was injected in mice either in an unconjugated form or cross-linked with cBSA. The quantity injected was standardized according to the amount of IgG present. A greater antibody response was obtained using the cBSA conjugate.

once it is added to a buffered solution. BSA remains soluble in the presence of up to 35% DMSO, becomes slightly cloudy at 40%, and precipitates at 45% (v/v).

Thyroglobulin and OVA

Thyroglobulin and ovalbumin (OVA) are used less often as carriers, but they are particularly valuable as nonrelevant carriers in ELISA tests designed to measure the antibody response after injection of an immunogen conjugate. Since an antibody response would be directed against both the carrier and the attached hapten, an ELISA done to quantify a specific antibody that only interacts with the hapten must not utilize the same carrier in the conjugate coated on the microplates. If the carrier conjugate used for the ELISA is identical to that used in the original immunization, the test results will be skewed by the contribution of carrier-specific antibodies. For this reason, a nonrelevant carrier—one that is not recognized by the products of the immune response—must be coupled with hapten and used for the ELISA test.

Since OVA and BSA possess some immunologically similar epitopes, a population of the antibodies produced against one often will cross-react against the other. Therefore, OVA cannot function as a nonrelevant carrier for BSA and vice versa. Either OVA or BSA, however, may be used as nonrelevant carriers for KLH, thyroglobulin, or the various toxoid proteins used as immunogen conjugates.

OVA makes up about 75% of the total protein in hen egg whites. The protein contains 20 lysine residues and 14 aspartic acid and 33 glutamic acid groups. This gives a total of 20 ε-amines, 1 N-terminal amine, 47 side-chain carboxylates, and 1 C-terminal carboxylate for conjugation reactions. The majority of acidic groups gives the protein a pI of 4.63. Additional sites of modification include 4-sulfhydryl groups, 10 tyrosines, and 7 histidine residues. OVA is sensitive to temperature (above 56°C), electric fields, and vigorous mixing. Care should be taken in handling the protein to prevent denaturation and subsequent precipitation.

One advantage of OVA is its extreme solubility characteristics in the presence of DMSO. A sparingly soluble hapten molecule may be dissolved in this solvent and added to an aqueous OVA reaction mixture to maintain solubility of the molecule during conjugation. OVA is soluble at up to 70% DMSO, becomes cloudy at 75%, and precipitates at 80% (v/v).

Thyroglobulin is a prohormone protein that is synthesized and stored in the thyroid gland. Specific proteolytic action on the protein *in vivo* causes the release of triiodothyronine and thyroxine, low-molecular-weight amino acid derivatives that affect metabolic rate and oxygen consumption. Thyroglobulin is a large, multisubunit protein composed of several polypeptide chains (MW 670,000). Its acidic pI (4.7) reflects the abundance of carboxylate groups. Thyroglobulin is also glycosylated, containing about 8–10% carbohydrate. Its use as an immunogen carrier protein is less frequent than that of KLH or BSA.

Tetanus and Diphtheria Toxoids

Toxoid proteins are biologically inactivated forms of native toxins. The most often used toxoid is tetanus toxoid, but diphtheria-derived toxoids and other proteins also are used occasionally (Anderson *et al.*, 1989). Tetanus toxoid (MW 150,000) has 106 amine groups, 10 sulfhydryls, 81 tyrosine residues, and 14 histidines that may participate in conjugation reactions with hapten molecules (Bizzini *et al.*, 1970). Diphtheria toxoid is derived from a protein secreted by certain strains of *Corynebacterium diphtheriae*. Its molecular weight is approximately 63,000 (Collier and Kandel, 1971). Both protein toxoids can be used to couple haptens through any of the chemical reactions described in this chapter. They generate strong immunological responses *in vivo*.

2.2. Liposome Carriers

Liposomes are artificial structures composed of phospholipid bilayers exhibiting amphiphilic properties (chapter 12). In complex liposome morphologies, concentric spheres or sheets of lipid bilayers are usually separated by aqueous regions that are sequestered or compartmentalized from the surrounding solution. The phospholipid constituents of liposomes consist of hydrophobic lipid tails connected to a head constructed of various glycerylphosphate derivatives. The hydrophobic interaction between the fatty acid tails is the primary driving force for creating liposomal bilayers in aqueous solutions.

The morphology of a liposome may be classified according to the compartmentalization of aqueous regions between bilayer shells. if the aqueous regions are sequestered by only one bilayer each, the liposomes are called unilamellar vesicles (ULV). If there is more than one bilayer surrounding each aqueous compartment, the liposomes are termed multilamellar vesicles (MLV). ULV forms are further classified according to their relative size, although rather crudely. Thus, there can be small unilamellar vesicles (SUV; usually less than 100 nm in diameter) and large unilamellar vesicles (LUV; usually greater than 100 nm in diameter). With regard to MLV, however, the bilayer structures cannot be easily classified due to the almost infinite number of ways each

bilayer sheet can be associated and interconnected with the next one. MLVs typically form large complex honeycomb structures that are difficult to classify or reproduce.

The overall composition of a liposome—its morphology, composition (including a variety of potential phospholipids and the degree of its cholesterol content), charge, and any attached functional groups—can affect the antigenicity of the vesicle *in vivo* (Allison and Gregoriadis, 1974; Alving, 1987; Therien and Shahum, 1989). When liposomes are used as carriers for immunization purposes, the haptens or antigens usually are attached covalently to the head groups using various phospholipid derivatives and cross-linking chemistries (Derksen and Scherphof, 1985). Most often these derivatization reactions are done off of phosphatidylethanolamine constituents within the liposomal mixture. The primary amine modification off the glycerylphosphate head group of phosphatidylethanolamine provides an ideal functional group for activation and subsequent coupling of hapten molecules (Shek and Heath, 1983). Stock preparations of activated liposomes may be prepared and lyophilized to be used as needed in coupling hapten molecules (Friede *et al.*, 1993). All of the amine-reactive conjugation methods discussed in this section may be used with phosphatidylethanolamine-containing liposomes; however, see chapter 12 for a more complete discussion of the unique considerations associated with conjugation of molecules to liposomes.

2.3. Synthetic Carriers

Synthetic molecules may be used as carriers if they are designed with the appropriate functional groups to couple hapten molecules. These carriers may consist of simple polymers such as poly-L-lysine, poly-L-glutamic acid, Ficoll, dextran, or polyethylene glycol (Lee *et al.*, 1980; Fok *et al.*, 1982; Boyle *et al.*, 1983; Hopp, 1984; Wheat *et al.*, 1985). Coupling of hapten molecules to the principal functional groups of these polymers can produce immunogenic conjugates that may be injected in animals to generate a specific antibody response.

Poly-L-lysine may be coupled to carboxylate-containing molecules using the carbodiimide conjugation procedure to yield amide linkages (Chapter 3, Section 1.1, and Chapter 9, Section 3). Homobifunctional or heterobifunctional cross-linking agents also may be used with poly-L-lysine, such as in the use of sulfo-SMCC (Chapter 9, Section 5). The polymer can be used as well for coupling hapten molecules and subsequent coating of microplates for ELISA procedures (Gegg and Etzler, 1993). Conversely, poly-L-glutamic acid may be coupled to amine-containing haptens by the same carbodiimide protocol. Ficoll and dextran carriers may be activated by mild sodium periodate oxidation to generate reactive aldehyde groups (Chapter 1, Section 4.4, and Chapter 15, Section 2.1). Coupling to amine-containing haptens then may be done by reductive amination (Chapter 3, Section 4). Polyethylene glycol chemical reactions involve alternate activation and coupling schemes that are addressed in Chapter 15, Section 1.

A unique synthetic molecule that can be used as a carrier is the so-called multiple antigenic peptide (MAP) (Tam, 1988; Posnett *et al.*, 1988). The MAP core structure is composed of a scaffolding of sequential levels of poly-L-lysine. The matrix is constructed from a divalent lysine compound to which two additional levels of lysine are attached. The final MAP compound consists of a symmetrical, octavalent primary

amine core to which hapten molecules may be attached. Coupling of up to eight peptide haptens to the MAP core yields a highly immunogenic complex having a molecular weight of typically greater than 10,000. The nature of the MAP carrier makes it ideal for remarkably defined conjugates useful in vaccine development.

One particularly novel carrier was reported to consist of 50- to 70-nm colloidal gold particles of the type often used in cytochemical labeling techniques for microscopy (Pow and Crook, 1993) (Chapter 14). Adsorption of peptide antigens onto gold and subsequent injection of the complex into rabbits in an adjuvant mixture resulted in rapid production of antibody of extremely high titer. The resultant antibodies could be used in immunocytochemistry at dilutions from 1 in 250,000 down to 1 in 1,000,000, which is orders of magnitude beyond the dilutions typically used with lower-titer antibodies.

3. Carbodiimide-Mediated Hapten–Carrier Conjugation

The coupling chemistry used to prepare an immunogen from a hapten and carrier protein is an important consideration for the successful production and correct specificity of the resultant antibodies. The choice of cross-linking methodology is governed by the functional groups present on both the carrier and the hapten as well as the orientation of the hapten desired for appropriate presentation to the immune system. An associated concern is the potential for antibody recognition and cross-reactivity toward the cross-linking reagent used to effect the conjugation. If antibodies are generated against the cross-linker bridge, then this may dilute the desired antibody response against the hapten. The use of a zero-length cross-linking procedure mediated by the water-soluble carbodiimide EDC eliminates this problem, since no bridging molecule is introduced between the hapten and the carrier.

The reactions involved in an EDC-mediated conjugation are discussed in Chapter 3, Section 1.1 [Note: EDC is 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride; MW 191.7]. The carbodiimide first reacts with available carboxylic groups on either the carrier or hapten to form a highly active *O*-acylisourea intermediate. The activated carboxylic group then can react with a primary amine to form an amide bond, with release of the EDC mediator as a soluble isourea derivative. The reaction is quite efficient with no more than 2 h required for it to go to completion and form a conjugated immunogen.

Since most peptide haptens contain either amines or carboxylic groups available for coupling, EDC-mediated immunogen formation may be the simplest method for the majority of hapten–carrier protein conjugations. Figure 275 shows the coupling of a carrier protein to a short peptide molecule through its amine terminus. It should be kept in mind, however, that this type of conjugation may occur at either the C- or the N-terminal of the peptide or at any carboxyl- or amine-containing side chains. Therefore, this method probably should be avoided if a particularly interesting part of the peptide contains groups that may be blocked or undergo coupling using the carbodiimide reaction. Also, when using peptides rich in Lys, Glu, Arg, His, or Asp, unacceptable crosslinking of the hapten may occur on conjugation, and thus change the antigenic structure of the resulting immunogen. However, some cross-linking or polymerization of the peptide on the surface of the carrier actually may be beneficial to the immunogenicity of the peptide, and thus create an even greater antibody response.

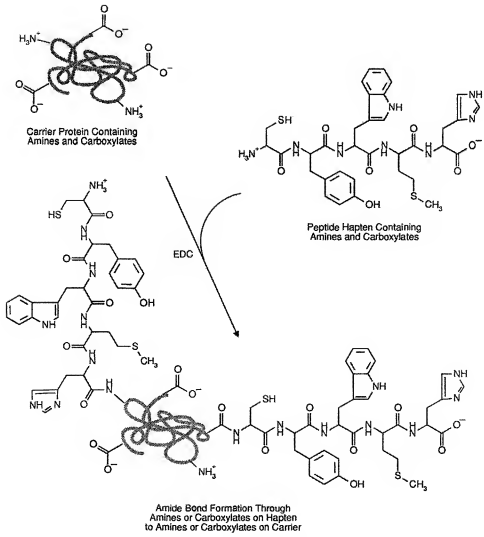


Figure 275 Peptide haptens are easily conjugated to carrier proteins using the water-soluble carbodiimide EDC.

Some investigators even advocate using no carrier protein when a peptide hapten is involved: merely polymerizing the peptide in the presence of EDC may result in a complex of high enough molecular weight to be immunogenic by itself. In general, EDC coupling is a very efficient, one-step method for forming a wide variety of peptide–carrier protein immunogens.

Figure 276 shows the results of an EDC conjugation study comparing a reaction done at pH 4.7 (A) to one done at pH 7.3 (B and C), with and without added sulfo-NHS (see Chapter 3, Section 1.2). The graphs show the elution profiles of a gel filtration separation after conjugation. In each case, a blank run done without the addition of EDC illustrates the separation of the protein carrier (the first peak) from

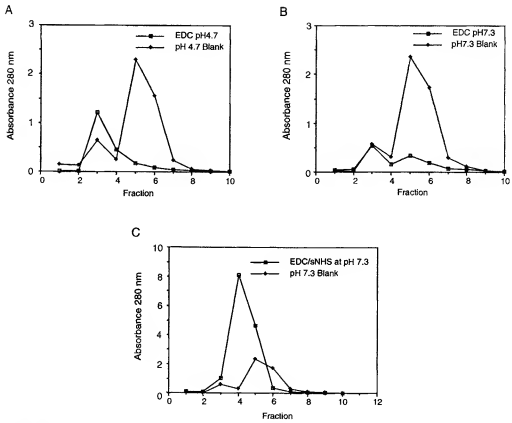


Figure 276 To assess the effectiveness of an EDC conjugation reaction of a peptide with a carrier protein, glycyl-tyrosine was coupled to BSA using various conditions. The graphs show a gel filtration profile on Sephadex G-25 after completion of the conjugation reaction. The first peak eluting off the column is the higher molecular weight carrier, while the second peak is excess peptide. The elution profiles demonstrate that the carbodiimide reaction proceeds with nearly equal efficiency at pH 4.7 (A) or pH 7.3 (B). In each graph, a comparison is shown between the separation of peptide and carrier without addition of EDC and the same mixture after reaction with EDC. Depletion of the peptide peak in the EDC-containing elution profiles indicates uptake of glycyl-tyrosine in the carrier conjugate. Some polymerization of peptide also is possible using this method, as evidence by movement of the peptide peak toward higher molecular weight elution points. Addition of sulfo-NHS to the reaction caused precipitation problems as well as obscuring the separation due to the absorbance of excess sulfo-NHS (C).

the lower molecular weight peptide and reagent peak (the second peak). Decrease in the peptide peak is indicative of successful conjugation. Complete recovery of the total absorbance at 280 nm usually does not occur, presumably due to a decrease in the peptide's absorptivity as it is conjugated or polymerized. Staros' method of adding sulfo-NHS to form an intermediate active ester that subsequently reacts with an amine to form the amide bond does not work as well due to excessive conjugation (causing precipitation in most cases) and interference from the eluting sulfo-NHS peak. The reaction proceeds with similar yields at either acid or neutral pH. Thus, the efficiency of an EDC conjugation reaction is approximately the same from pH 4.7 to physiological conditions.

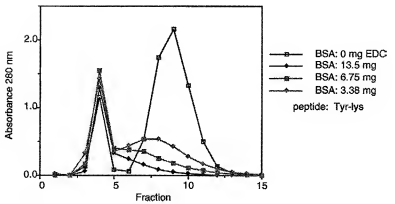


Figure 277 To study the conjugation of peptides to carriers using different levels of EDC, tyrosyl-lysine was conjugated to BSA and separated after the reaction by chromatography on a Sephadex G-25 column. As the EDC level was increased in the reaction, more peptide reacted and the peptide peak (the second peak) was depleted. The absorbance of the carrier peak (the first one) increases as more peptide is conjugated.

Figure 277 shows the result of the conjugation of the dipeptide tyrosyl-lysine to BSA using various concentrations of EDC. Again, the elution profile shows the gel filtration pattern resulting after the reaction. The first peak is the protein carrier while the second is the peptide. Progressive decrease in the peptide peak with increasing amounts of EDC added to the reaction mixture correlates to increased conjugation yields. A side reaction to EDC conjugation of haptens that contain both an amine and a carboxylate group is hapten polymerization. This is revealed in the movement of the peptide peak toward higher molecular weights (e.g., decreased time of elution) with increasing amounts of EDC added to the reaction.

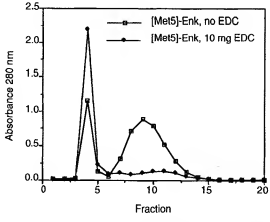


Figure 278 Conjugation of the biological peptide [Met5]-enkephalin to BSA using EDC. The graph shows the gel filtration profile (on Sephadex G-25) after completion of the conjugation reaction. A blank run with no added EDC was done to illustrate the peak absorbances that would be obtained if no conjugation took place. With addition of 10 mg of EDC to a reaction mixture consisting of 2 mg of BSA plus 2 mg of peptide, nearly complete conjugate formation was obtained.

Figure 278 illustrates the conjugation of [Met5]-enkephalin with BSA using EDC. The gel filtration profile after cross-linking reveals that the peptide peak effectively disappears upon complete conjugation with the carrier protein. With nicely soluble peptides such as this one, the immunogen remains freely soluble even at high modification levels. For less soluble peptides or haptens, reducing the amount of EDC addition may be necessary to maintain solubility in the conjugate.

To illustrate the similarity of an EDC conjugation reaction using a different carrier protein, but the same peptide, Fig. 279 shows the gel filtration separation after conjugation of [Met5]-enkephalin to OVA. The uptake of peptide on addition of EDC is almost identical to that observed when conjugating to BSA. This is logical, since on a per mass basis, there is very little difference between these proteins in the amount of amines or carboxylates available for conjugation.

Figure 280 shows the conjugation of tyrosyl-lysine to KLH using various concentrations of EDC. The elution profile shows the gel filtration pattern resulting after the reaction. Progressive decrease in the peptide peak (peak 2) with increasing amounts of EDC correlates to increased conjugation (or polymerization) yields. Despite the extremely high molecular weight of KLH compared to the other commonly used carriers, the conjugation reaction using EDC again proceeds with results virtually identical to those of the similar study shown in Fig. 277 using BSA as the carrier. In fact, superimposing the two studies on the same graph demonstrates the reproducibility of an EDC-facilitated reaction (Fig. 281).

Due to the high molecular weight of KLH and its solubility characteristics, the conjugation of this protein to some haptens can result in precipitation of the complex. This is especially true if the level of EDC addition is similar to the EDC concentrations used with lower molecular weight carriers such as BSA or OVA. Figure 282 shows the elution profile resulting from the gel filtration separation of KLH and the peptide [Met5]-enkephalin after an EDC reaction. To result in a soluble immunogen, only 0.1 to 0.2 times the amount of EDC was added, compared to similar BSA or OVA conjuga-

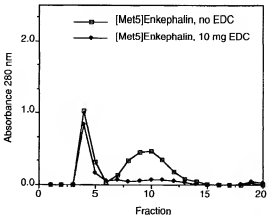


Figure 279 To illustrate the consistency of an EDC-mediated reaction, [Met5]-enkephalin was conjugated to ovalbumin using conditions identical to those described for BSA in Fig. 278. Note the similarity in the degree of conjugate formation.

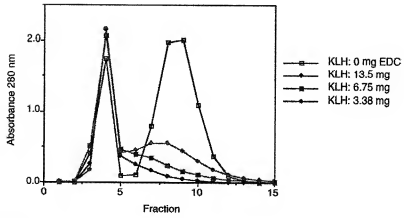


Figure 280 The conjugation of tyrosyl-lysine to KLH is illustrated by the gel filtration pattern on Sephadex G-25 after the reaction. The first peak is the carrier protein and the second peak is the peptide. A blank containing no EDC is also shown to provide baseline peak heights that would be obtained if no cross-linking occurred. When more EDC was added, more peptide was conjugated, as evidenced by peptide peak depletion.

tion reactions. Even at levels this low, however, the coupling of peptide to the carrier is very efficient and results in an excellent immunogen.

These studies using EDC-facilitated conjugations were done to develop an optimal protocol for the preparation of immunogens by carbodiimide cross-linking. For haptens (i.e., peptides) that display good solubility in aqueous solution, the level of reagent addition should result in a soluble immunogen conjugate. When using haptens that are sparingly soluble or insoluble in aqueous environments, the conjugation reaction may result in a precipitated complex. Precipitation often can be controlled by scaling back

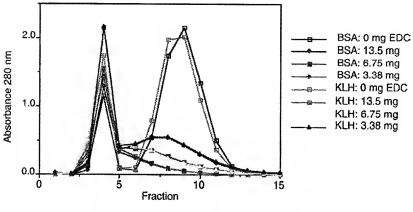


Figure 281 EDC conjugation reactions can be extraordinarily consistent using the same peptide cross-linked to two carrier proteins. This figure shows the gel filtration pattern on Sephadex G-25 after completion of the cross-linking reaction. Conjugation of tyrosyl-lysine to BSA and KLH are shown. The first peaks represent eluting carrier, while the second peaks are the excess peptide. Note the consistency of conjugation using the same levels of EDC addition.

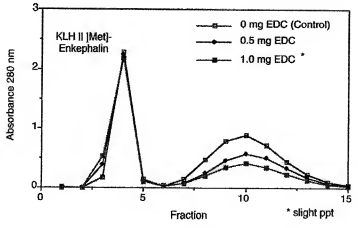


Figure 282 Conjugation to KLH often can cause precipitation due to the high molecular weight of the carrier protein. The conjugation of [Met5]-enkephalin to KLH yields a soluble immunogen if the level of EDC addition is about 0.1 times that typically used with BSA as a carrier. The figure shows the gel filtration pattern on Sephadex G-25 after completion of the cross-linking reaction. The first peak is KLH and the second peak is excess peptide. Depletion of the peptide peak correlates to hapten–carrier conjugation.

the level of EDC addition or limiting the time of the reaction. If a precipitated immunogen is not a problem (most precipitated, high-molecular-weight conjugates are very immunogenic), then the following protocol is applicable to the great majority of peptide–carrier protein conjugations. Pierce Chemical offers a kit containing all the reagents necessary for an EDC-mediated hapten–carrier conjugation.

Protocol

1. Dissolve the carrier protein in 0.1 *M* MES, 0.15 *M* NaCl, pH 4.7, at a concentration of 10 mg/ml. If using KLH, increase the NaCl concentration of all buffers to 0.9 *M* (yes, 0.9 *M*, not 0.9%) to maintain solubility of the protein. For neutral pH conjugations, substitute 0.1 *M* sodium phosphate, 0.15 *M* NaCl, pH 7.2, for the MES buffer.
2. Dissolve up to 4 mg of the peptide or hapten to be coupled in 1 ml of the reaction buffer chosen in step 1. If the peptide to be coupled is already in solution, it may be used directly if it is in a buffer containing no other amines or carboxylic acids and is at a pH between 4.7 and 7.2. Note: If an assessment of the degree of peptide coupling is desired, measure the absorbance at 280 nm of the 1 ml peptide solution before proceeding to step 3. In some cases, a dilution of the peptide solution may be necessary to keep the absorbance on scale for the spectrophotometer. If the peptide is sparingly soluble in aqueous solution, it may be dissolved in DMSO and an aliquot added to the carrier solution. See the previous discussion on carrier proteins to determine the levels of DMSO compatible with carrier protein solubility.
3. Add 500 µl of the peptide solution to 200 µl of carrier protein. For greater reaction volumes, keep the ratio of peptide/carrier addition the same and proportionally scale up the amount of EDC added in the next step. If the peptide is

initially dissolved in DMSO, much less peptide volume compared to protein volume should be used to maintain solubility (see discussion in step 2).

4. For conjugations using relatively low-molecular-weight proteins, such as BSA or OVA, add the peptide/carrier solution to a vial containing 10 mg of EDC (Pierce) and gently mix to dissolve. For high-molecular-weight KLH immunogens, first dissolve one vial containing 10 mg of EDC in 1 ml of deionized water, and immediately transfer 50 µl of this solution to the carrier/peptide solution. Gently mix.
5. Allow the reaction to continue at room temperature for 2 h.

Note: Although the conjugation protocols have been optimized by preparing a number of different peptide–carrier conjugates, some peptide sequences or other haptens may cause precipitation of the carrier upon coupling. This may occur as a result of changing the carrier's solubility characteristics through surface modification or due to polymerization. A small amount of precipitation is not a problem and can easily be removed by centrifugation before the gel filtration step. If severe precipitation occurs, however, the amount of EDC added to the reaction may have to be scaled back to eliminate or reduce it. With BSA or OVA conjugates, this may mean using as little as 1–3 mg of EDC instead of the recommended 10 mg. With KLH as a carrier, reducing the EDC levels to 0.1 mg may be necessary.

6. Purify the hapten–carrier conjugate by gel filtration or dialysis.

4. NHS Ester-Mediated Hapten–Carrier Conjugation

Hapten–carrier conjugation may be accomplished by the use of homobifunctional reagents containing NHS ester groups on both ends. The active esters are highly reactive toward amine functional groups on proteins and other molecules to form stable amide bonds. Cross-linking agents of various lengths may be used for this conjugation strategy, including the sulfo-NHS ester analogs, which are more water-soluble than the NHS esters without a sulfonic acid group (Chapter 4, Section 1).

Using homobifunctional NHS esters, amine-containing haptens may be conjugated to amine-containing carriers in a single step (Fig. 283). The carrier is dissolved in a buffer having a pH of 7–9 (0.1 M sodium phosphate, pH 7.2 works well). The hapten molecule is added to this solution at a suitable molar excess to assure multipoint attachment of the hapten to the carrier. A molar excess of 20–30 times that of the carrier concentration is a good starting point. Next, the NHS ester cross-linker is added to the solution to provide at least a threefold molar excess over that of the hapten. For cross-linkers insoluble in aqueous solution, first solubilize them in DMF or DMSO at higher concentration, and then add an aliquot of this stock solution to the hapten–carrier solution. The conjugation reaction is complete within 2 h at room temperature. Some adjustment of the level of hapten and cross-linker addition may be necessary to avoid extensive precipitation of the conjugate, especially when using rather hydrophobic hapten molecules.

Another method of NHS ester mediated hapten–carrier conjugation is to create reactive sulfo-NHS esters directly on the carboxylates of the carrier protein using the EDC/sulfo-NHS reaction described in Chapter 3, Section 1.2. A carbodiimide reaction in the presence of sulfo-NHS activates the carboxylate groups on the carrier protein to form amine-reactive sulfo-NHS esters. The activation reaction is done at pH

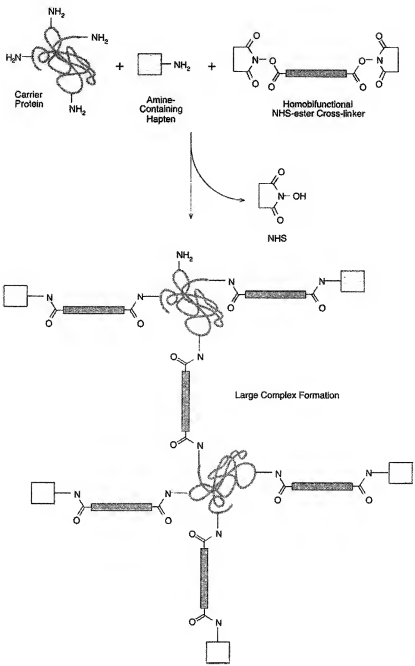


Figure 283 Hapten–carrier immunogen conjugates can be formed using homobifunctional NHS ester cross-linkers. The reaction may create large polymeric complexes, some of which could precipitate.

6, since the amines on the protein will be protonated and therefore be less reactive toward the sulfo-NHS esters that are formed. In addition, the hydrolysis rate of the esters is dramatically slower at acid pH. Thus, the active species may be isolated in a

reasonable time frame without significant loss in conjugation potential. To quench unreacted EDC, 2-mercaptoethanol is added to form a stable complex with the remaining carbodiimide, according to Carraway and Triplett (1970). In the following protocol, a modification of the Grabarek and Gergely (1990) two-step method, sulfo-NHS is used instead of NHS so that active ester hydrolysis is slowed even more (Thelen and Deuticke, 1988; Anjaneyulu and Staros, 1987). Subsequent conjugation with amine-containing hapten molecules yields hapten–carrier conjugates created by amide bond formation (Fig. 284).

Protocol

1. Dissolve the carrier protein to be activated in 0.05 *M* MES, 0.5 *M* NaCl, pH 6 (reaction buffer), at a concentration of 1 mg/ml.
2. Add to the solution in step 1 a quantity of EDC and sulfo-NHS (both from Pierce) to obtain a concentration of 2 m*M* EDC and 5 m*M* sulfo-NHS. To aid in aliquoting the correct amount of these reagents, they may be quickly dissolved in

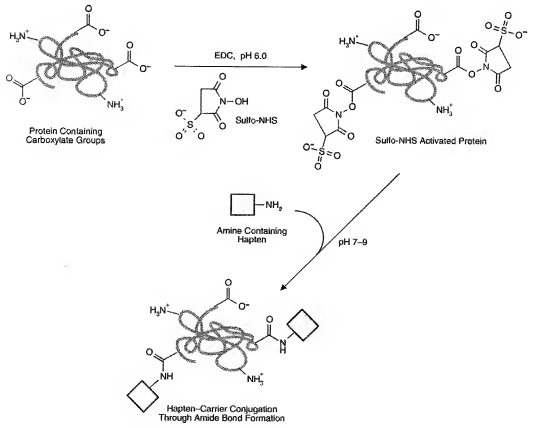


Figure 284 The carbodiimide EDC can be used in the presence of sulfo-NHS to create reactive sulfo-NHS ester groups on a carrier protein. Subsequent coupling with an amine-containing hapten can be done to create amide bond linkages.

water at a higher concentration, and then immediately a volume pipetted into the protein solution to obtain the proper molar quantities.

3. Mix and react for 15 min at room temperature to form the sulfo-NHS esters.
4. Add 2-mercaptoethanol to the reaction solution to obtain a final concentration of 20 mM. Mix and incubate for 10 min at room temperature. Note: if the protein being activated is sensitive to this level of 2-mercaptoethanol, instead of quenching the reaction chemically, the activation may be terminated by desalting (see step 5).
5. If the reaction was quenched by the addition of 2-mercaptoethanol, the activated protein may be added directly to an amine-containing hapten molecule for conjugation. Alternatively, or if no 2-mercaptoethanol was added, the activated protein may be purified from reaction by-products by gel filtration using Sephadex G-25 or equivalent. The desalting operation should be done rapidly to minimize hydrolysis and recover as much of the active ester functional group as possible. The use of centrifugal spin columns of some sort may afford the greatest speed in separation. After purification, add the activated protein to the hapten for conjugation. The hapten molecule should be dissolved in 0.1 *M* sodium phosphate, pH 7.5.
6. React for at least 2 h at room temperature.
7. Remove excess reactants by gel filtration or dialysis.

5. NHS Ester–Maleimide Heterobifunctional Cross-linker-Mediated Hapten–Carrier Conjugation

A common method for coupling haptens to carrier proteins involves the use of a heterobifunctional cross-linker containing an NHS ester and a maleimide group. This type of cross-linker allows better control over the coupling process than homobifunctional or zero-length conjugation methods by incorporating a two- or three-step reaction strategy directed against two different functional targets. In this approach, the carrier protein first is activated with the cross-linker through its amine groups, purified to remove excess reactants, and then cross-linked to a hapten molecule containing a sulfhydryl group. One of the most useful reagents for this conjugation approach is sulfo-SMCC.

The reactions associated with a sulfo-SMCC conjugation are shown in Fig. 285 [note: sulfo-SMCC is sulfosuccinimidyl-4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate; MW, 436.37] (see Chapter 5, Section 1.3). This crosslinking reagent mediates the conjugation of a carrier protein through its primary amine groups to a peptide or other hapten through sulfhydryl groups. The active *N*-hydroxysulfosuccinimide ester (sulfo-NHS) end of sulfo-SMCC first is reacted with available primary amine groups on the carrier protein. This reaction results in the formation of an amide bond between the protein and the cross-linker with the release of sulfo-NHS as a by-product. The carrier protein is then isolated by gel filtration to remove excess reagents. At this stage, the purified carrier possesses modifications generated by the cross-linker resulting in a number of reactive maleimide groups projecting from its surface. The maleimide portion of sulfo-SMCC is a thiol-reactive group that can be used in a secondary step to conjugate with a free sulfhydryl (i.e., a cysteine residue) on a peptide or other hapten, resulting in a stable thioether bond.

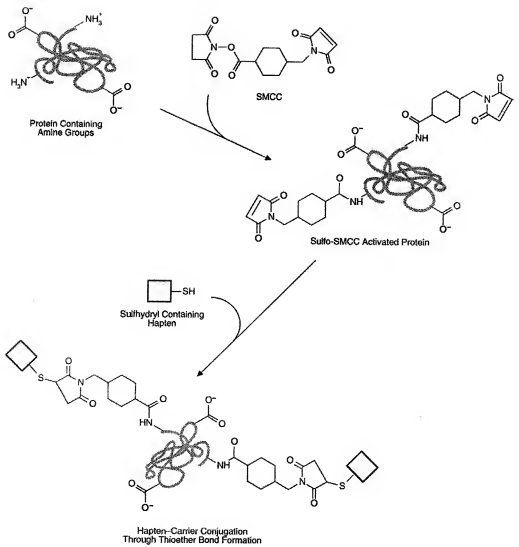


Figure 285 A common way of conjugating sulfhydryl-containing haptens to carrier proteins is to activate the carrier with sulfo-SMCC to create an intermediate maleimide derivative. The maleimide groups then can be coupled to thiols to form thioether bonds.

The use of sulfo-SMCC over the other common maleimide-containing cross-linkers such as MBS or SMPB provides the advantages of water solubility during the activation step and increased stability of the maleimide group prior to conjugation with a peptide. The improved stability ensures that the majority of the maleimide groups substituted on the carrier will survive the subsequent purification process without degradation. The relatively good stability of the maleimide group of sulfo-SMCC is probably due to the neighboring steric effects of its cyclohexane ring. The faster hydrolysis rates of other maleimide-type cross-linkers can be a significant problem, since they readily break down to the maleamic acid form, which is no longer reactive toward sulfhydryls (Fig. 286).

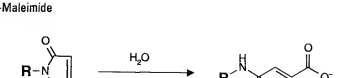


Figure 286 A maleimide group may hydrolyze in aqueous solution to an open maleamic acid form that is unreactive with sulfhydryls.

A disadvantage to using SMCC or other NHS–maleimide-type cross-linkers with hindered ring structures (such as MBS) is the relatively high immunogenicity of the cross-bridge. Studies have shown that a hapten–carrier complex formed from such cross-linkers generates significant antibody response against the spacer group itself, not just the hapten and carrier. To minimize the antibody population directed against the cross-bridge of the conjugate, the use of aliphatic straight-chain spacers will exhibit the lowest immunogenicity (Peeters *et al.,* 1989). Although SMCC (or sulfo-SMCC) is used in the following protocol, substitution of GMBS (or sulfo-GMBS) (Chapter 5, Section 1.7) will limit the immune response to the cross-linker.

Since many peptides do not naturally contain cysteine residues with free sulfhydryls, a terminal cysteine may be incorporated during peptide synthesis, or where appropriate, disulfide groups may be reduced to generate them. Alternatively, a thiolating reagent such as 2-iminothiolane (Traut's reagent) can be used to modify existing amino groups and introduce a sulfhydryl (see Chapter 1, Section 4.1). Caution must be taken when using this last technique, however, because multiple sites of modification may alter the immunogenic structure of the hapten.

If a terminal cysteine residue is added to a peptide during its synthesis, its sulfhydryl group provides a highly specific conjugation site for reacting with a sulfo-SMCC-activated carrier. All peptide molecules coupled using this approach will display the same basic conformation after conjugation. In other words, they will have a known and predictable orientation, leaving the majority of the molecule free to interact with the immune system. This method therefore can preserve the major epitopes on a peptide while still enhancing the immune response to the hapten by being covalently linked to a larger carrier protein. In addition, the well-known chemical reactivity of a sulfo-SMCC-mediated immunogen preparation permits covalent conjugation in a controllable fashion that can be highly defined for quality assurance purposes.

The process of carrier activation by sulfo-SMCC may be followed by performing a simple purification step after the reaction using Sephadex G-25. Figure 287 shows the gel filtration profiles for the separation of sulfo-SMCC-activated BSA and OVA. The first peak of both separations represents the elution point for the carrier protein, while the absorbance due to reaction by-products of the crosslinker is contained in the second peak (shown only as its leading edge). Activated proteins exhibit an increase in their absorbance at 280 nm over an identical sample with no added sulfo-SMCC due to their covalently attached maleimide groups. After isolation, the activated protein may be frozen and lyophilized to preserve maleimide coupling activity toward sulfhydryl-containing haptens. Pierce Chemical sells a number of maleimide-activated carrier proteins in lyophilized form for easy hapten conjugation.

After a carrier protein has been activated with sulfo-SMCC it is often useful to

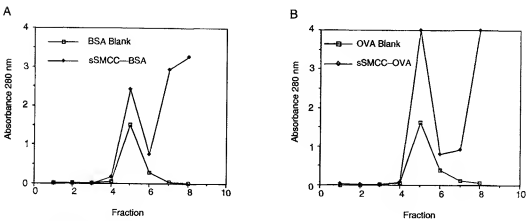


Figure 287 Carrier proteins may be activated with sulfo-SMCC to produce maleimide derivatives reactive with sulfhydryl-containing molecules. The graphs show the gel filtration separation on Sephadex G-25 of maleimide-activated BSA (A) and OVA (B) after reaction with sulfo-SMCC. The first peak is the protein and the second peak is excess cross-linker. The maleimide groups create increased absorbance at 280 nm in the activated proteins.

measure the degree of maleimide incorporation prior to coupling an expensive hapten. Ellman's reagent may be used in an indirect method to assess the level of maleimide activity of sulfo-SMCC-activated proteins and other carriers. First, a sulfhydryl-containing compound such as 2-mercaptoethanol or cysteine is reacted in excess with the activated protein. The amount of unreacted sulfhydryls remaining in solution then is determined using the Ellman's reaction (Chapter 1, Section 4.1). Comparison of the response of the sample to a blank reaction using the native, nonactivated protein at

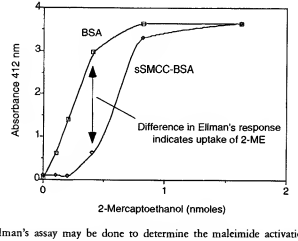


Figure 288 An Ellman's assay may be done to determine the maleimide activation level of SMCC-derivatized proteins. Reaction of the activated carrier with different amounts of 2-mercaptoethanol results in various levels of sulfhydryls remaining after the reaction. Detection of the remaining thiols using an Ellman's assay indirectly indicates the amount of sulfhydryl uptake into the activated carrier. Comparison of the Ellman's response to the same quantity of 2-mercaptoethanol plus an unactivated carrier indicates the absolute amount of sulfhydryl that reacted. Calculation of the maleimide activation level then can be done.

the same concentration and a series of standards made from a serial dilution of the sulfhydryl compound employed in the assay gives the amount of sulfhydryl compound conjugated and thus an estimate of the original maleimide activity.

Figure 288 shows a plot of the results of such an assay done to determine the maleimide content of activated BSA. This particular assay used 2-mercaptoethanol, which is relatively unaffected by metal-catalyzed oxidation. For the use of cysteine or cysteine-containing peptides in the assay, however, the addition of EDTA is required to prevent disulfide formation. Without the presence of EDTA at 0.1 *M*, the metal contamination of some proteins (especially serum proteins such as BSA) is so great that disulfide formation proceeds preferential to maleimide coupling. Figure 289 shows a similar assay for maleimide-activated BSA using the more innocuous cysteine as the sulfhydryl-containing compound.

Using this type of cysteine-uptake assay, it is possible to determine the percentage of maleimides that reacted over time. Thus, an indication of the reaction efficiency of a sulfhydryl-containing compound coupling with a maleimide-activated protein may be determined. Figure 290 shows the reaction rate for the coupling of cysteine to maleimide-activated BSA. Note that maximal coupling is obtained in less than 2 h, with over 80% yield in under 30 min.

The following protocol describes the activation of a carrier molecule with sulfo-SMCC and its subsequent conjugation with a hapten. The preactivated carriers containing maleimide groups ready for coupling to a sulfhydryl-containing compound are now commercially available in a stable freeze-dried form (Pierce Chemical). Substitution of GMBS (Chapter 5, Section 1.7) in the following protocol will provide a straight-chain aliphatic spacer with less immunogenicity than the ring structure of SMCC's cross-bridge.

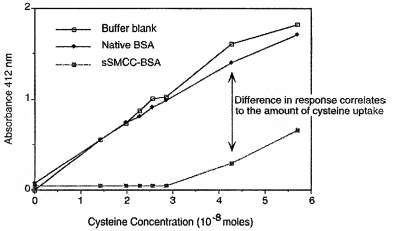


Figure 289 Cysteine also may be used in an Ellman's assay to determine the maleimide activation level of SMCC-derivatized proteins. Reaction of the activated carrier with different amounts of cysteine results in various levels of sulfhydryls remaining after the reaction. The coupling must be done in the presence of EDTA to prevent metal-catalyzed oxidation of sulfhydryls. Detection of the remaining thiols using an Ellman's assay indirectly indicates the amount of sulfhydryl uptake into the activated carrier. Comparison of the Ellman's response to the same quantity of cysteine plus an unactivated carrier indicates the absolute amount of sulfhydryl that reacted. Calculation of the maleimide activation level then can be done.

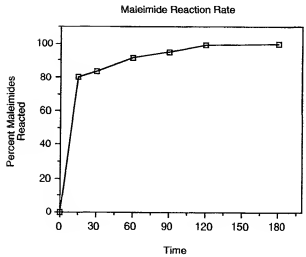


Figure 290 The rate of reaction of cysteine with maleimide-activated BSA was determined using an Ellman's assay for remaining sulfhydryl groups after the reaction, according to Fig. 289. Nearly all of the available maleimides are coupled with sulfhydryls within 2 h.

Protocol

1. Dissolve the carrier of choice at a concentration of 10 mg/ml in 0.1 *M* sodium phosphate, 0.15 *M* NaCl, pH 7.2 (activation buffer). Note: For use of KLH, increase the NaCl concentration to 0.9 *M*.
2. Dissolve sulfo-SMCC (Pierce) at a concentration of 10 mg/ml in the activation buffer. Immediately transfer the appropriate amount of this cross-linker solution to the vial containing the dissolved carrier protein.

Note: The amount of cross-linker solution to be transferred is dependent on the level of activation desired. Suitable activation levels can be obtained for the following proteins by adding the indicated quantities of the sulfo-SMCC solution. The degree of sulfo-SMCC modification often determines whether the carrier will maintain solubility after activation and coupling to a hapten. KLH, in particular, is sensitive to the amount of cross-linker addition. KLH usually retains solubility at about 0.1–0.2 times the mass of cross-linker added to BSA. This level of addition still results in excellent activation yields, since KLH is significantly larger than most of the other protein carriers.

Add the following quantities of sulfo-SMCC solution to each ml of carrier protein solution:

a. BSA, 500 µl
b. cBSA, 200 µl
c. OVA, 500 µl
d. KLH, 100 µl

Carriers having molecular weights similar to that of BSA or OVA may be activated at the same level with good success. Cationized BSA requires less cross-linker addition due to its greater quantity of amines present.

3. React for 1 h at room temperature.
4. Immediately purify the activated carrier protein by gel filtration using a volume of Sephadex G-25 equal to 15 times the volume of the activation reaction. To perform the chromatography use 0.1 *M* sodium phosphate, 0.15 *M* NaCl (0.9 *M* for KLH), 0.1 *M* EDTA, pH 7.2 (conjugation buffer). The EDTA is present to prevent metal-catalyzed sulfhydryl oxidation to disulfides. This is a particular problem when using BSA due to contaminating iron from hemolysis. Concentrations less than 0.1 *M* EDTA will not fully inhibit the oxidation reaction, especially if a cysteine containing peptide is to be conjugated to the activated carrier. Apply the sSMCC/carrier reaction mixture to the column while collecting 0.5- to 1-ml fractions. Pool the fractions containing the activated carrier (the first peak to elute from the column) and discard the fractions containing excess sulfo-SMCC (the second peak). The activated carrier should be used immediately or freeze-dried to maintain maleimide stability.
5. Dissolve a sulfhydryl-containing hapten or peptide to be conjugated at a concentration of 10 mg/ml in the conjugation buffer. Other hapten concentrations may be used, depending on its solubility. If an excess of the peptide solution is made at this time, an estimate of the degree of conjugation may be determined later (see section below). Add this solution to the pooled fractions containing the activated carrier at an equivalent mass ratio (1 mg hapten/mg of the carrier). Alternatively, the peptide may be added in solid form directly to the activated carrier solution if it is known to be freely soluble and can be weighed out in the appropriate quantity.
6. Allow the conjugation reaction to proceed for 2 h at room temperature.
7. The hapten–carrier conjugate now may be used for injection purposes without further purification.

An estimate of the degree of conjugation may be made by assaying the amount of sulfhydryl present before and after the coupling reaction. A portion of the peptide solution before mixing with the activated carrier should be saved to compare with the reaction mixture after the conjugation is complete. The comparison is made using a solution of Ellman's reagent (5,5′-dithiobis-(2-nitrobenzoic acid), which reacts with sulfhydryls to form a highly colored chromophore having an absorbance maximum at 412 nm ($\varepsilon_{412nm} = 1.36 \times 10^4\ cm^{-1}\ M^{-1}$) (Chapter 1, Section 4.1). A generalized procedure is presented here. Modifications to this guideline may have to be made for each individual peptide to obtain the appropriate response to the Ellman's reagent.

1. Using a microtiter plate (96-well) dispense 200 μl of 0.1 *M* sodium phosphate, 0.15 *M* NaCl, 0.1 *M* EDTA, pH 7.2 (conjugation buffer), into each well to be used.
2. Add 10 μl of the peptide solution before conjugation to the appropriate wells in duplicate.
3. Add 10 μl of the reaction mixture after the conjugation reaction is complete to another set of wells in duplicate.
4. Add 20 μl of Ellman's reagent (1 mg/ml dissolved in the gel filtration purification buffer) to each well including one containing only buffer (220 μl) to use as a blank.
5. Incubate for 15 min at room temperature.
6. Measure the absorbance of all wells using a microplate reader with a filter set at 410 nm.

A comparison of the blank corrected values before and after conjugation should give an indication of the percentage of peptide coupled. To be more quantitative, a standard curve must be run to focus in on the linear response range of the peptide–Ellman's reaction. Using cysteine as a representative sulfhydryl compound (similar in Ellman's response to a peptide having one free sulfhydryl) it is possible to obtain very accurate determinations of the amount that coupled to the activated carrier. Figure 289, discussed previously in this section, shows the results of this type of assay.

6. Active-Hydrogen-Mediated Hapten–Carrier Conjugation

Conjugation chemistry for the coupling of haptens to carrier molecules is fairly well defined for compounds having common functional groups to facilitate such attachment. The types of functional groups generally useful for this operation include easily reactive components such as primary amines, carboxylic acids, aldehydes, or sulfhydryls.

However, for hapten molecules containing no easily reactive functional groups, conjugation can be difficult or impossible using current techniques. To solve this problem, demanding organic synthesis is frequently required to modify the hapten molecule to contain a suitable reactive portion. Particularly, certain drugs, steroidal compounds, dyes, or other organic molecules often have structures that contain no available "handles" for convenient cross-linking.

Frequently, these difficult to conjugate compounds do have certain sufficiently active hydrogens that can be reacted with a carrier molecule using specialized reactions designed for this purpose. This section describes two choices for this conjugation problem, the diazonium procedure and the Mannich reaction. Both of them are able to cross-link haptens through any available active hydrogen to carrier molecules, resulting in immunogens suitable for injection.

6.1. Diazonium Conjugation

Diazonium coupling procedures have been used for many years in organic synthesis and for cross-linking or immobilization of active hydrogen-containing compounds (Inman and Dintzis, 1969; Cuatrecasas, 1970). Diazonium derivatives can couple with haptens containing available phenolic or, to a lesser extent, imidazole groups in an electrophilic substitution reaction (Riordan and Vallee, 1972). They also may undergo minor secondary reactions with sulfhydryl groups and primary amines (Glazer *et al.*, 1975).

The most important reaction of a diazonium group, however, is with available tyrosine and histidine residues within peptide haptens, rapidly creating diazo linkages. This method of conjugation is especially useful for site-directed cross-linking of tyrosine-containing peptides. Since tyrosine usually is present only in limited quantities in a given peptide, use of diazonium conjugation can cross-link and orient all peptide molecules in an identical fashion on the carrier. The result is excellent reproducibility in preparation of the immunogen, and a consistent presentation of the peptide on the surface of the carrier to the immune system for antibody production.

Derivatives of carbohydrate antigens also have been coupled to carrier proteins

through the use of an intermediate diazonium group (McBroom *et al.*, 1976). In this case, an aminophenyl glycoside was prepared by reaction of the reducing end of the oligosaccharide with β-(*p*-aminophenyl)ethylamine and then formation of the diazotized derivative with sodium nitrite (Zopf *et al.*, 1978a). On mixing with carrier proteins containing tyrosine residues, the carbohydrate derivative is coupled via a diazo bond.

Creation of a diazonium group on phenolic compounds or tyrosine side-chain groups is possible by forming an intermediate nitrophenol derivative. Reaction of tyrosine-containing proteins and peptides with tetranitromethane effectively nitrates the ring in the *ortho* position (Vincent *et al.*, 1970). Reduction of the nitro group to an amine then is done using sodium dithionite (sodium hydrosulfite; $Na_2S_2O_4$) (Sokolovsky *et al.*, 1967; Chapter 1, Section 4.3). The aminophenol derivative finally is reacted with sodium nitrite in acidic conditions to form the highly reactive diazonium group (Fig. 291). Once treated, the diazonium compound must be added immediately to the conjugation reaction, since the species is extremely unstable in aqueous environments.

The active diazonium typically is a colored compound, sometimes orange, dark brown, or even black in concentrated solutions. The conjugated immunogen therefore usually is deeply colored as well. The coupling reaction is performed at alkaline pH, optimally at pH 8 for histidinyl residues and pH 9–10 for tyrosine groups. In practice, however, it is not possible to target a histidine group in the presence of a tyrosine group.

Diazo linkages are reversible bonds that may be cleaved by addition of 0.1 *M*

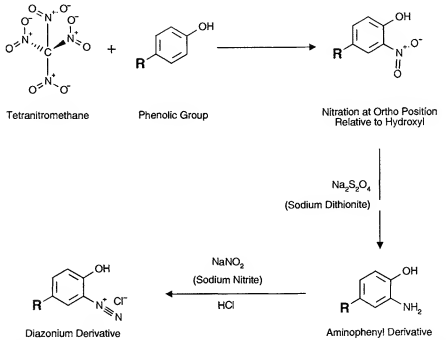


Figure 291 Phenolic compounds may be derivatized to contain reactive diazonium groups by nitration with tetranitromethane followed by reduction with sodium dithionite and diazotization with sodium nitrite in dilute HCl.

sodium dithionite in 0.2 *M* sodium borate, pH 9. Release of the cross-links can be followed by loss of the diazo bond color.

A simple, one-step conjugation reaction is possible with diazonium chemistry if a *bis*-aminophenyl compound is used as a homobifunctional cross-linking agent. Activation of the aminophenyl groups with sodium nitrite creates the requisite *bis*-diazonium derivative that can couple with active hydrogen-containing haptens and carriers. In this way, tyrosine-containing peptides can be conjugated with tyrosine-containing carrier proteins in a single step. Compounds useful for this procedure include o-tolidine and benzidine (Chapter 4, Section 9), both of which contain aromatic amines that easily can be diazotized (Fig. 292).

From a practical perspective, however, any of the conjugation methods utilizing

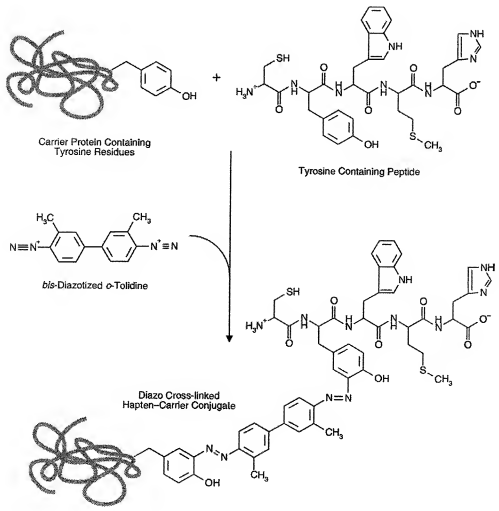


Figure 292 The conjugation of a tyrosine-containing carrier protein and a tyrosine-containing peptide may be done using *bis*-diazotized tolidine to form diazo cross-links.

diazonium chemistry can be brimming with problems. The rate of reaction of the diazonium species is so rapid that much of the total coupling potential can be lost through intramolecular cross-linking. As the diazonium groups are formed they may immediately cross-link to the active hydrogens present on the aminophenyl precursor molecules, even before addition of a second molecule to be conjugated. Even without addition of a second active-hydrogen-containing compound, the diazonium-activated molecule will turn brown to black within an hour, indicating formation of diazo bonds and self-conjugation. For this reason, the reproducibility of conjugation reactions using this method usually is poor.

The following protocol describes the use of diazotized o-tolidine for the cross-linking of active-hydrogen-containing haptens to active-hydrogen-containing carriers. Using a *bis*-diazonium compound is perhaps the simplest method of conjugation, but as in many one-step cross-linking procedures, it often results in some precipitation of the final product. Reaction conditions may have to be adjusted to prevent severe precipitation; however, even an insoluble immunogen can be useful in generating an antibody response.

Caution: Both o-tolidine and benzidine are potential carcinogens. Protective clothing, gloves, and the use of a fume hood are recommended. Avoid all contact of the compounds with skin or clothing and do not inhale vapors or dust.

Protocol

1. Diazotization of *o*-tolidine: Weigh out 25 mg of o-tolidine and place in a small test tube or vial. Add 4.5 ml of 0.2 *N* HCl and mix to dissolve. Chill the solution on ice. Dissolve 17.5 mg of sodium nitrite into 0.5 ml of ice-cold deionized water, and add it to the vial containing the *o*-tolidine. The solution should begin to turn an orange color, progressively getting darker as the reaction continues. React for 1 h on ice, mixing periodically. At the completion of the diazotization reaction, aliquots of the solution may be stored at −20°C.
2. Dissolve 10 mg of carrier protein into 0.5 ml 0.15 *M* sodium borate, 0.15 *M* NaCl, pH 9.
3. Dissolve 5–10 mg of a peptide hapten containing at least one tyrosine residue per milliliter of 0.15 *M* sodium borate, 0.15 *M* NaCl, pH 9.
4. Mix 0.5 ml of the peptide solution with 0.5 ml of the carrier protein solution. Chill on ice. Add 0.4 ml of the *bis*-diazotized tolidine solution. There should be a color change from orange to red almost immediately. Continue the reaction for 2 h on ice in the dark.
5. Purify the conjugate by gel filtration or dialysis using PBS, pH 7.4. The preparation is now ready for immunization purposes.

6.2. Mannich Condensation

Another approach for cross-linking haptens to carriers when the hapten has no available common functional groups (amines, carboxylates, sulfhydryls, etc.), but does possess active hydrogens, is to use the Mannich reaction. Using this strategy an active hydrogen-containing compound can be condensed with formaldehyde and an amine in the Mannich reaction, resulting in a stable alkylamine linkage. Particularly, com-

pounds containing replaceable hydrogens provided by the presence of certain activating chemical constituents can be aminoalkylated using this reaction (see Chapter 2, Section 5.4, and Chapter 4, Section 6.1, for additional information on active hydrogens).

Formally, the Mannich reaction consists of the condensation of formaldehyde (or sometimes another aldehyde) with ammonia, in the form of its salt, and another compound containing an active hydrogen. Instead of using ammonia, however, this reaction can be done with primary or secondary amines, or even with amides. An example is illustrated in the condensation of acetophenone, formaldehyde, and a secondary amine salt (the active hydrogens are shown underlined):

$$C_6H_5COC\underline{H}_3 + CH_2O + R_2NH{\cdot}HCl \rightarrow C_6H_5COCH_2CH_2NR_2{\cdot}HCl + H_2O$$

The Mannich reaction provides a viable alternative to the diazonium conjugation method (discussed previously), because of the disadvantages inherent in the instability of both the diazonium group and the resultant diazo linkage. By contrast, conjugations done through Mannich condensations result in stable covalent bonds.

The cross-linking scheme using this method can make use of the native ε- and N-terminal amines on carrier proteins as the source of primary amine for the condensation reaction. Added to the conjugation reaction is formaldehyde and the desired hapten to be coupled containing an appropriately active hydrogen.

To increase the yield of conjugated hapten using this procedure, cationized BSA is used as the carrier protein in the method described below (Section 2.1). The greater density of amine groups on cBSA available for participation in the Mannich reaction over that available on native proteins provides better results in coupling active hydrogen-containing haptens.

One note of caution should be realized when using the Mannich reaction. The hapten to be coupled should not contain any amine groups or hapten polymerization may occur preferential to conjugation to the carrier. For instance, when performing site-directed coupling of tyrosine-containing peptides through their phenolic side chain, the diazonium reaction should be used instead of the Mannich procedure, otherwise peptide-to-peptide coupling may occur.

Protocol

1. In a vial or test tube are placed and mixed:
   a. 200 μl of a solution containing 10 mg/ml cationized BSA (cBSA, Pierce) in 0.1 *M* MES, 0.15 *M* NaCl, pH 4.7 (coupling buffer). The acidic conditions of this coupling buffer are optimal for the Mannich reaction.
   b. 200 μl of a solution consisting of 10 mg/ml of a hapten containing an active hydrogen. The solution can be made up in absolute ethanol in the case of water insoluble haptens and is made up in coupling buffer in the case of water-soluble haptens.
   c. 50 μl of additional absolute ethanol in the case of water-insoluble haptens.
   d. 50 μl of 37% formaldehyde (Sigma) solution. Caution: Use a fume hood and avoid contact or inhalation of vapors.

2. Incubate the reaction mixture in a water bath or oven at a temperature of 37–57°C for a period of 3–24 h.

3. To separate unconjugated haptens and formaldehyde from the synthesized conjugate, apply the entire volume of reactants to a Sephadex G-25 desalting column containing a bed volume of at least 10 times the volume of reactants. PBS, pH 7.2, can be used for the desalting step. The purified conjugate is recovered in the void volume.

The yield of conjugation using the Mannich reaction is dependent on the reactivity of active hydrogens within the hapten molecule. It is often difficult to predict the relative reactivity of any given compound in this reaction. Thus, trial and error may be necessary to determine the suitability of the Mannich procedure.

Figure 293 shows the conjugation reaction of the dye phenol red to cBSA using the Mannich reaction. The active hydrogens which participate in the conjugation are *ortho* to the hydroxyl group on the phenol ring. After purification of the conjugate by gel filtration to remove any unconjugated dye and formaldehyde, a wavelength scan was done to assess the degree of conjugate formation. Figure 294 shows the results of this scan. The protein solution appeared red after conjugation and desalting, indicating successful cross-linking had occurred.

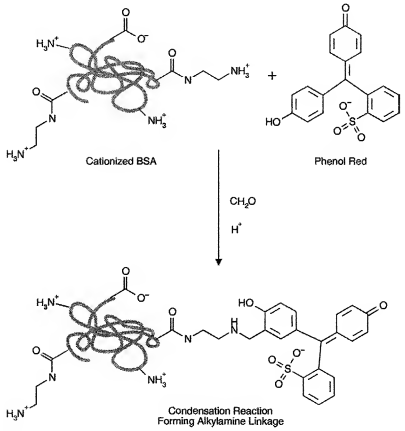


Figure 293 The conjugation of phenol red to cationized BSA using the Mannich reaction.

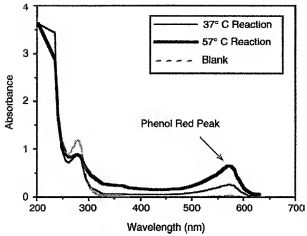


Figure 294 Absorbance scan comparing unconjugated cBSA with the same carrier that had been coupled with phenol red using the Mannich reaction. Two different reaction times are compared, indicating that extended reactions yield increased conjugate formation.

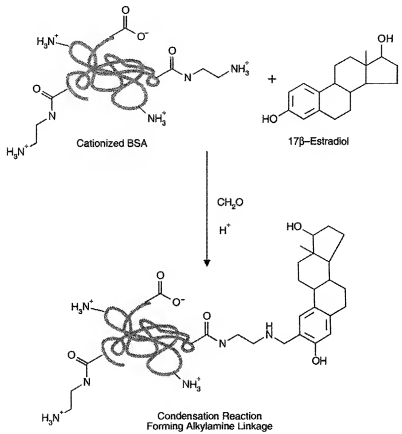


Figure 295 The conjugation of estradiol to cBSA using the Mannich reaction.

The steroidal compound 17β-estradiol was also conjugated to cBSA using the Mannich reaction. Similar to phenol red, conjugation with estradiol occurs *ortho* to the hydroxyl group on its phenolic ring (Fig. 295). After purification of the conjugate by gel filtration, it was injected in mice intraperitoneally using alum as adjuvant. Antibodies were successfully produced against the coupled estradiol. Controls consisting of unconjugated estradiol with and without mixed carrier molecules also were injected, but resulted in no antibody production.

7. Glutaraldehyde-Mediated Hapten–Carrier Conjugation

The homobifunctional cross-linking reagent glutaraldehyde can be used in a one- or two-step conjugation protocol to prepare hapten–carrier conjugates. Glutaraldehyde can react with primary amine groups to create Schiff bases or double-bond (Michael-type) addition products (Chapter 4, Section 6.2). The Schiff base intermediate may form resonance-stabilized products with the α,β-unsaturated aldehydes of the glutaraldehyde polymers predominating at basic pH values (Monsan *et al.*, 1975; Peters and Richards, 1977; Korn *et al.*, 1972). One such product, a quaternary pyridinium complex, can form as a cross-link between two lysine residues (Chapter 1, Section 4.4). Reduction of the Schiff bases with sodium borohydride or sodium cyanoborohydride yields stable secondary amine linkages.

The reaction of glutaraldehyde with protein carriers and peptide haptens involves mainly lysine ε-amine and N-terminal α-amine groups. The conjugates formed are usually of high molecular weight and may cause precipitation products. In addition, the orientation of the hapten on the carrier is indiscriminate with oligomers of the peptide predominating. However, despite the disadvantages of using glutaraldehyde-mediated cross-linking, it still remains one of the most popular techniques for creating bioconjugates.

There are several different protocols commonly used in the literature to form glutaraldehyde conjugates. Some methods utilize a neutral pH environment in phosphate buffer (pH 6.8–7.5) while others use more alkaline conditions in carbonate buffer (pH 8–9) (Price *et al.*, 1993). In general, the higher pH conditions will more effectively form Schiff base intermediates and result in greater conjugation yields, but also higher molecular weight conjugates. The concentration of glutaraldehyde in the reaction medium generally varies from 0.20 to 1% (Avrameas, 1969; Avrameas and Ternynck, 1969; Jeanson *et al.*, 1988; Ford *et al.*, 1978) with occasional use of very dilute solutions (0.05%). The lower concentrations of glutaraldehyde generate lower yields of conjugation and result in less stable conjugates (Briand *et al.*, 1985).

The following procedure utilizes the one-step glutaraldehyde method. A two-step method may be used to limit somewhat polymerization of the conjugate (Chapter 10, Section 1.2). Varying the pH and the amount of glutaraldehyde added to the reaction can control the yield and molecular weight of the conjugates formed.

Protocol

1. Dissolve the carrier protein (or another carrier that contains amine groups) in 0.1 *M* sodium carbonate, 0.15 *M* NaCl, pH 8.5, at a concentration of 2 mg/ml.
2. Add peptide hapten to the carrier solution to obtain a concentration of about 2

mg/ml. Alternatively, determine the molar ratio of peptide to carrier. Ratios of 20:1 to 40:1 (peptide:carrier) usually result in good immunogens.

3. Add fresh glutaraldehyde to the peptide/carrier solution to obtain a 1% final concentration. Mix well. Caution: Use of a fume hood is recommended when working with glutaraldehyde. Avoid contact with skin and clothing. Do not breathe vapors.
4. React for 2–4 h at 4°C. Periodically mix the solution or use a gentle rocker.
5. The conjugate may be stabilized by addition of a reductant such as sodium borohydride or sodium cyanoborohydride. Usually sodium cyanoborohydride is recommended for specific reduction of Schiff bases, but since the conjugate has already formed at this point, the use of sodium borohydride will both reduce the associated Schiff bases and eliminate any remaining aldehyde groups. Add sodium borohydride to a final concentration of 10 mg/ml. Continue to react for 1 h at 4°C.
6. Purify the conjugate by gel filtration using Sephadex G-25 or dialysis to remove excess reagents. The presence of high-molecular-weight conjugates may cause some precipitation in the final product. If turbidity is evident, instead of gel filtering, dialyze against PBS, pH 7.4.

8. Reductive-Amination-Mediated Hapten–Carrier Conjugation

Hapten molecules containing aldehyde residues may be cross-linked to carrier molecules by use of reductive amination (chapter 3, Section 4). At alkaline pH values, the aldehyde groups form intermediate Schiff bases with available amine groups on the carrier. Reduction of the resultant Schiff bases with sodium cyanoborohydride or sodium borohydride creates a stable conjugate held together by secondary amine bonds.

Oligosaccharide haptens are especially amenable for coupling to carriers by reductive amination. Carbohydrate molecules may contain reducing ends that can be utilized for this purpose (Chapter 1, Section 2.1) (Gray, 1978), or aldehyde residues may be specifically created from other functional groups (Chapter 1, Section 4.4). Often, mild oxidation using sodium periodate can be used to cleave adjacent diols on sugar residues, forming reactive aldehyde groups (Anderson *et al.*, 1989).

If the reducing ends of oligosaccharide molecules are used for this technique, then the reaction time necessary to obtain good yields of hapten–carrier conjugates may be from several days to several weeks. The extended reaction period is due to the limited time reducing sugars are in their open, aldehydic form (usually far less than 1% of the available saccharide at any given time). By contrast, if periodate oxidized carbohydrate is used, then the reaction time is reduced to only hours. It should be noted, however, that extensive periodate oxidation could modify antigenic determinants and no longer reflect the native structure and characteristics of the carbohydrate.

Protocol

1. Dissolve the carrier protein at a concentration of 10 mg/ml in 0.1 *M* sodium phosphate, 0.15 *M* NaCl, pH 8.
2. Add the aldehyde-containing oligosaccharide to the carrier solution at a concentration sufficient to obtain at least a 20-fold molar excess of hapten to carrier.

Adding a much greater molar excess of oligosaccharide to couple through reducing ends (i.e., up to 200-fold excess) will help to drive the conjugation reaction to completion.

3. Add sodium cyanoborohydride (Aldrich) to a concentration of 20 mg/ml. *Caution:* Highly toxic! Use a fume hood and avoid inhalation of dust or vapors. Seal the reaction vessel with parafilm. Do not use a rigid sealing cap, since cyanoborohydride will liberate hydrogen gas bubbles over time and may rupture the vessel.
4. React at room temperature with periodic mixing. Reaction times can vary significantly depending on the reactivity of the aldehyde group. For coupling of the reducing ends of polysaccharide molecules, continue the reaction for at least 100 h. High-density derivatization through the reducing ends may take up to 2 weeks. For coupling of periodate oxidized carbohydrate, where the aldehyde residues are more accessible, the reaction is complete within 4 h.
5. Purify the hapten–carrier conjugate to remove excess reductant by gel filtration or dialysis using a PBS, pH 7.4, buffer. Removal of unconjugated carbohydrate may be more difficult. If the oligosaccharide was of high molecular weight so that the unconjugated carbohydrate cannot be easily separated from the conjugate using typical desalting gels or small-porosity dialysis tubing, then a gel filtration matrix possessing greater exclusion limits may be used. However, often it is not necessary to remove unconjugated hapten from such preparations.